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Application#	Patent#	Status	Date Filed	Title	Inventor Name
<u>07691559</u>	Not Issued	166	04/25/1991	ANTIGEN RECOGNIZED BY ANTIBODIES ASSOCIATED WITH PARANEOPLASTIC OPSOCLONUS AND METHODS OF USE THEREOF	DARNELL , ROBERT B.
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<u>06409391</u>	4409195	150	08/19/1982	PURIFICATION OF SILICON SOURCE MATERIALS	DARNELL , ROBERT D.
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<u>09923284</u>	Not Issued	020	08/06/2001	METHODS AND AGENTS FOR INDUCING APOPTOSIS AND METHODS FOR THEIR IDENTIFICATION	DARNELL, ROBERT B.
<u>60336249</u>	Not Issued	020	11/15/2001	METHOD AND IDENTIFICATION OF DOWNSTREAM mRNA LIGANDS TO FMRP AND THEIR ROLE IN FRAGILE X SYNDROME AND ASSOCIATED DISORDERS	DARNELL, ROBERT B.

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Interactions of viruses with dendritic cells: a double-edged sword.

Bhardwaj N.

J Exp Med. 1997 Sep 15;186(6):795-9.

The Rockefeller University, New York 10021, USA.
bhardwn@rockvax.rockefeller.edu

Cytotoxic T lymphocyte priming versus cytotoxic T lymphocyte tolerance induction: a delicate balancing act involving dendritic cells.

Melief CJ, Schoenberger S, Toes R, Offringa R.

Haematologica. 1999 Jun;84 Suppl EHA-4:26-7.

Dendritic cells resurrect antigens from dead cells.

Larsson M, Fonteneau JF, Bhardwaj N.

Trends Immunol. 2001 Mar;22(3):141-8.

The Rockefeller University, New York, NY 10021, USA.

Antigens that do not normally access the cytoplasm of antigen-presenting cells, such as certain tumor and viral antigens, become targets of cytotoxic T lymphocytes (CTLs). Over the past 25 years, substantial evidence has emerged for an 'exogenous' pathway for loading MHC class I molecules. Dendritic cells are potent stimulators of T-cell responses and can induce CD8(+) CTLs by phagocytosis of dead tumor or virus-infected cells. Here, Marie Larsson and colleagues discuss the role of dendritic cells in stimulating MHC class I-restricted T-cell responses by exogenous routes.

Mobilizing dendritic cells for tolerance, priming, and chronic inflammation.

Sallusto F, Lanzavecchia A.

J Exp Med. 1999 Feb 15;189(4):611-4.

Comment on:

J Exp Med. 1999 Feb 15;189(4):627-36 PMID: 9989977

Dendritic cells, interleukin 12, and CD4+ lymphocytes in the initiation of class I-restricted reactivity to a tumor/self peptide.

Grohmann U, Fioretti MC, Bianchi R, Belladonna ML, Airoldi E, Surace D, Silla S, Puccetti P.

Crit Rev Immunol. 1998;18(1-2):87-98.

Department of Experimental Medicine, University of Perugia, Italy.

Cell-mediated immunity involving CD8+ lymphocytes is effective in mediating rejection of murine mastocytoma cells bearing P815AB, a tumor-associated and self antigen showing similarity to tumor-specific shared antigens in humans. Although this antigen may act as an efficient target for class I-restricted responses in immunized mice, neither P815AB expressed on tumor cells nor a related synthetic nonapeptide will activate unprimed CD8+ cells for in vivo reactivity, measured by skin test assay. We review evidence showing that the failure of P815AB to initiate CD8+ cell reactivity may be due to defective recruitment of accessory and Th1-like cells to the afferent phase of the response initiated by transfer of mice with dendritic cells pulsed in vitro with the P815AB peptide. Although the copresence of a T helper peptide in dendritic cell priming in vitro with P815AB may compensate for the poor generation of accessory and Th1 cells in the adoptively transferred mice, recombinant IL-12 can replace the helper peptide in both effects. Effective priming to P815AB in vivo is achieved by either exposing dendritic cells to IL-12 prior to P815AB priming or administering the recombinant cytokine in vivo. Different approaches suggest that IL-12 may act both on accessory cells to improve presentation of previously undescribed class II-restricted epitopes of P815AB and on CD4+ cells to improve recognition of such epitopes. In particular, at the CD4+ cell level, IL-12 apparently acts as an adjuvant and an inhibitor of anergy induction. These data offer useful information for developing vaccination strategies using dendritic cells and class I-restricted tumor peptides in humans.

Dendritic cells and tolerance induction.

Steptoe RJ, Thomson AW.

Clin Exp Immunol. 1996 Sep;105(3):397-402.

Pittsburgh Transplantation Institute, University of Pittsburgh, PA 15213,
USA.

Dendritic cells (DC) are widely accepted as the most potent antigen-presenting cells (APC), and considerable interest has been generated in their potential for the immunological therapy of cancer and infectious disease. Recently, however, a broader understanding of the phenotypic diversity and functional heterogeneity of DC has been acquired. Thus, in addition to having a role in central tolerance, DC are now regarded as potential modulators of peripheral immune responses. Harnessing this potential may offer a new approach to the immunosuppressive therapy of allograft rejection or autoimmunity. Here, the concept of "tolerogenic" DC is placed in the context of rapidly accumulating new evidence of the diverse properties of these important APC.

Dendritic cells as a tool to induce anergic and regulatory T cells.

Jonuleit H, Schmitt E, Steinbrink K, Enk AH.

Trends Immunol. 2001 Jul;22(7):394-400.

Comment in:

Trends Immunol. 2001 Oct;22(10):546-7 PMID: 11702722

Dept of Dermatology, University of Mainz, Langenbeckstr. 1, D-55131 Mainz, Germany.

The induction of antigen-specific T-cell tolerance in the thymus and its maintenance in the periphery is crucial for the prevention of autoimmunity. As well as their stimulatory functions, there is growing evidence that dendritic cells, acting as professional antigen-presenting cells, also maintain and regulate T-cell tolerance in the periphery. This control function is exerted by certain maturation stages and subsets of different ontogeny, and can be influenced by immunomodulatory agents. What is the current state of knowledge of the "immunoregulatory" properties of dendritic cells and how might tolerance-inducing dendritic cells be relevant to therapeutic applications in humans?

Manipulation of dendritic cells for tolerance induction in
transplantation and autoimmune disease.

Lu L, Thomson AW.

Transplantation. 2002 Jan 15;73(1 Suppl):S19-22.

Thomas E. Starzl Transplantation Institute, Department of Surgery,
University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania 15213,
USA. Lul@msx.upmc.edu

Dendritic cells (DC) constitute a complex system of uniquely well-equipped antigen-presenting cells that initiate and regulate immune responses. Extensive recent studies have improved our understanding of DC development, differentiation, activation, and function. DC exist as distinct subsets that differ in their lineage affiliation, surface molecule expression, and biological function. These factors seem to determine the T-cell polarizing signals and type of T cell response-T helper 1, T helper 2, or T regulatory- induced by DC (1). Evidence has accumulated that DC play an important role in both central and peripheral tolerance via various mechanisms, including induction of T-cell anergy, immune deviation, T regulatory cell activity, and promotion of activated T-cell apoptosis. Although many of the details of the molecular basis of DC tolerogenicity have yet to be elucidated, emerging information suggests that costimulatory molecule deficiency, expression of death-inducing ligands (in particular Fas [CD95] ligand), microenvironmental factors (in particular anti-inflammatory/immunosuppressive cytokines), and inhibition of gene transcription regulatory proteins (e.g., nuclear factor-kappaB) can impart tolerogenic potential to DC (2). Manipulation of DC by control of their maturation and differentiation, or genetic engineering of these cells to express immunosuppressive molecules, offers potential for therapy of allograft rejection and autoimmune disease. In this brief overview, we outline principles and methods for generation of "tolerogenic" DC and outcomes that have been reported in experimental models. Space constraints limit literature citations.

Designer dendritic cells for tolerance induction: guided not misguided missiles.

Hackstein H, Morelli AE, Thomson AW.

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PMID: 11473833 [PubMed - indexed for MEDLINE]

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Transplant Proc. 1999 Nov;31(7):2738-9. No Abstract Available.

PMID: 10578270 [PubMed - indexed for MEDLINE]

From PubMed

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Are dendritic cells the key to liver transplant tolerance?

Thomson AW, Lu L.

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PMID: 10081227 [PubMed - indexed for MEDLINE]

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Imai Y, Yamakawa M.

Pathol Int. 1996 Nov;46(11):807-33.

PMID: 8970191 [PubMed - indexed for MEDLINE]

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1. Transplantation:
2003 Jan 15, 75(1):137-145
2002 Jan 15, 73 (1 suppl):S19-S22
2. Blood, 2003 Feb 15, 101(4):1439-1445
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2001 Oct, 22(10):546-547
2001 Mar, 22(3):141-148
4. Pathol Int, 1996 Nov, 46(11):807-833
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8. Haematologica, 1999 Jun, 84 Suppl EHA-4, pp: 26-27
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10. Nature Immunology, 2001 Nov, 2(11):1010-1017

thanks!

Mobilizing Dendritic Cells for Tolerance, Priming, and Chronic Inflammation

By Federica Sallusto and Antonio Lanzavecchia

From the Basel Institute for Immunology, CH-4005 Basel, Switzerland

Migration of Dendritic Cells from Blood to Tissues. A fundamental aspect of dendritic cell (DC) function is their capacity to migrate. It allows them to exert a continuous surveillance for incoming antigens in almost all body tissues and a prompt report to T cells in secondary lymphoid organs. Under steady state conditions, the rate of DC migration from blood to tissues and from tissues to lymph nodes is probably very low, and most of these DCs reside in the tissues in a dormant state ready to be activated by pathogens. However, in inflammatory conditions the rate of DC migration can be considerably increased to meet the increased requirement for antigen presentation. Several recent papers shed new light into the mechanisms that control the various steps that DCs have to undergo to perform their function in the induction of tolerance, priming, and chronic inflammation.

To exit from the blood stream DCs, like other leukocytes, first need to tether to the endothelium (Fig. 1). This process, which is essential for the subsequent steps of firm adhesion and extravasation, is mediated by selectins that bind to specific carbohydrates on specialized cell surface molecules (1). In this issue, Robert et al. demonstrate that DCs that circulate in peripheral blood express a glycosylated form of P-selectin glycoprotein ligand (PSGL)-1 that binds to P- and E-selectins, which are expressed at low levels on endothelial cells and are upregulated by inflammatory stimuli (2). Using an *in vivo* imaging system, they show that DCs tether and roll on P- and E-selectins expressed by capillary endothelial cells and preferentially extravasate at sites of inflammation. This novel finding indicates that blood-borne DCs are poised to exit blood at inflammatory sites, thus allowing rapid recruitment of these APCs where their surveillance function is most needed.

Recruitment of leukocytes from blood to tissues is regulated at the level of the endothelial cells, where inflammatory cytokines increase the expression of adhesion molecules and chemokines and induce the formation of Weibel-Palade bodies. These represent storage granules for P-selectin (3, 4) and, as shown in two recent papers, also for IL-8 (5, 6). The content of the Weibel-Palade bodies can be rapidly mobilized by stimulation of endothelial cells with histamin or thrombin. This mechanism can be viewed as a form of "memory," since it allows endothelial cells that had been exposed to inflammatory stimuli to respond to a new challenge rapidly and without need for new protein synthesis, which has clear physiological as well as immunopathological significance.

A long-standing question has been the nature of the circulating cells that give rise to tissue DCs. Are these the few cells with DC phenotype characteristics that are present in the blood stream, or are there more frequent precursors that rapidly develop into DCs after entry into tissues? Monocytes have been shown to exit blood (7), and represent nonproliferating precursors that differentiate *in vitro* to immature DCs in cultures supplemented with GM-CSF and IL-4 (8). The factors that may promote the monocyte to DC transition *in vivo* still need to be defined. Recently, Randolph et al. showed that in an *in vitro* culture system, human peripheral blood monocytes cultured with endothelial cells differentiate into DCs within 2 d, particularly after phagocytosing particles in subendothelial collagen (9). These DCs migrated back across the endothelium as they

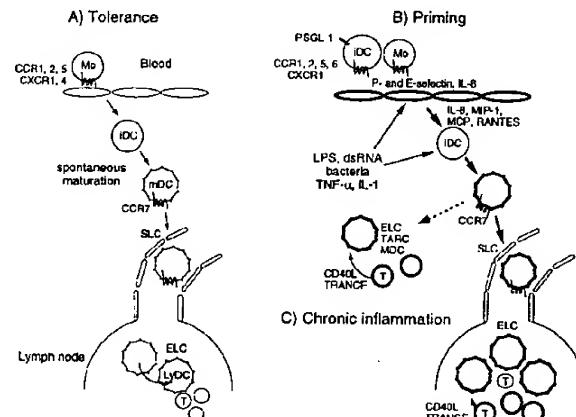


Figure 1. Mobilization and activation of DCs control the induction of tolerance, priming, and chronic inflammation. (A) Under steady state conditions, the recruitment of DC precursors into tissues and the migration of mature DCs to lymph nodes occur at low rates. Tissue antigens carried by short-lived migratory DCs may be transferred to resident lymphoid DCs that induce T cell tolerance. (B) Activated endothelial cells recruit DCs at higher rates. Immature DCs are attracted by inflammatory chemokines towards the site of inflammation, where bacterial and viral products induce DC maturation and activation. In the lymph node, activated T cells can further enhance DC activation and survival via CD40L and TRANCE. Because many and highly activated DCs are present, a productive T cell response is induced. (C) Mature DCs that fail to migrate to lymph nodes may serve as nucleation sites for chronic inflammatory reaction. Chemokines produced by these DCs attract maturing DCs as well as recently activated T cells, that maintain the inflammatory reaction. Thick lines indicate activated cells. Mo, monocytes; iDC, immature DCs; mDC, mature DCs; lymphoid DCs.

would do during entry into lymphatics, while those that remained in the subendothelial matrix became macrophages. These results suggest that the interaction with endothelial cells or extracellular matrix may be sufficient to drive differentiation of monocytes to either DCs or macrophages, each possessing inherently different migratory capacity. It will be interesting to apply the *in vivo* imaging system used by Robert et al. (2) to trace monocyte migration and differentiation *in vivo*.

The Role of Chemokines in the Multistep Navigation of DCs. Chemokines play a role not only in the process of extravasation, but also in the subsequent process of migration within the tissues to the final target. Different chemokines provide codes for areas that are undergoing different types of inflammatory reactions or are sites of constitutive traffic (10, 11). A current view holds that the whole migration process occurs in distinct steps, each driven by a particular chemokine–chemokine receptor pair (12). DCs provide a striking example of this so-called multistep navigation, since they use different sets of chemokine receptors to migrate from blood to inflamed tissues and from there to the lymphatics by which they reach their final destination in the T cell areas of lymph nodes (Fig. 1).

Monocyte and monocyte-derived immature DCs express receptors for inflammatory chemokines such as CCR1, CCR2, CCR5, and CXCR1, and are therefore attracted to inflammatory sites where cognate ligands such as IL-8, macrophage inflammatory protein (MIP)-1 α , monocyte chemotactic protein (MCP)-1, and regulated upon activation, normal T cell expressed and secreted (RANTES) are produced (13, 14). Monocytes also express CXCR4, which may be responsible for low level constitutive recruitment by stromal cell-derived factor (SDF)-1 (13). In addition, immature DCs present in tonsils and in cultures derived from CD34 $^{+}$ progenitors express CCR6, a receptor specific for liver and activation-regulated chemokine (LARC)/MIP-3 α , which is produced in the crypts of tonsils (15). Altogether it appears that inflammatory chemokines guide the first step of DC migration from blood into tissues and within tissues to inflammatory sites where antigens must be captured.

Besides being attracted by inflammatory chemokines, DCs also produce these chemokines in large amounts. The production of inflammatory chemokines by DCs is rapidly triggered after exposure to various maturation stimuli, and has two major consequences. First, it enhances recruitment of immature DCs, which is important to sustain antigen sampling at later time points. Second, it downregulates the expression of the cognate receptors on maturing DCs, thus allowing them to leave the inflamed tissues (13).

The second step of DC migration, from inflamed tissues into lymphatics and from there to T cell areas of lymph nodes, is regulated by a different set of chemokines. Maturing DCs upregulate receptors for constitutive chemokines such as CXCR4, CCR4, and especially CCR7 (13–15). CCR7 recognizes secondary lymphoid tissue chemokine (SLC), produced by lymphatic endothelial cells (16), and Epstein-Barr virus-induced molecule 1 ligand chemokine

(ELC), produced in the T cell areas by mature DCs (13, 15, 17), and therefore appears to be a key receptor for this second step of directional migration (Fig. 1).

Although at early time points after induction of maturation DCs are abundant sources of inflammatory chemokines, at later time points mature DCs produce high levels of constitutive chemokines such as thymus and activation-regulated chemokine (TARC), macrophage-derived chemokine (MDC), and ELC (F. Sallusto, unpublished data). This allows mature DCs, once they reach the T cell areas, to attract other maturing DCs as well as naive and recently activated T cells that express the cognate receptors CCR7 and CCR4 (for a review, see reference 18). Therefore, chemokine production by mature DCs plays an important function in organizing T cell areas and lymph node structure.

Migration of Stimulatory or Tolerizing DCs. Antigen presentation can lead either to full activation and priming of effector and memory T cells or to an abortive stimulation, resulting in functional inactivation and T cell tolerance. Finding the factors that regulate the balance between tolerance and response is now considered the holy grail of immunology. The current view is that the critical factor lies in the nature of the APC: mature DCs prime, whereas resting B cells and tissue cells tolerate. This notion has been recently challenged by Kurts and colleagues (19). These authors show that, in the absence of inflammation, a model antigen expressed in tissue cells is presented by bone marrow-derived APCs on class I molecules in the draining lymph node. Strikingly, this presentation results in transient CD8 T cell proliferation, which leads to tolerance. These observations imply that migratory, bone marrow-derived APCs (most likely DCs) pick up antigen in noninflamed tissues and migrate at low rate to the lymph nodes to induce tolerance to tissue antigens. At odds with the current view, the findings by Kurts et al. suggest that DCs are responsible not only for priming, but also for tolerance.

Two mechanisms may account for this striking finding. The first possibility is that the tolerogenic DCs represent a specialized lineage. A likely candidate is the lymphoid DC described by Shortman and colleagues, which can stimulate T cells but appears to be unable to support IL-2 production (20). If distinct DC subsets are involved in induction of tolerance or response, it will be important to assess whether they are recruited in response to different stimuli. For instance, tolerogenic DCs may be mobilized constitutively, whereas the stimulatory DC lineage may be preferentially recruited under inflammatory conditions. Recently, Inaba and colleagues showed that antigens carried by short-lived migratory DCs can be transferred to lymph node-resident, most likely lymphoid DCs (21; Fig. 1). They suggested that, in the absence of inflammation, this mechanism may be responsible for the induction of tolerance to tissue antigens, thus accounting for the observation of Kurts et al. (19).

The second possibility is that the same DC type is responsible for inducing either tolerance or response, depending on several factors such as the number of DCs that reach the lymph node, their life span, and the nature and amount of costimulatory molecules and cytokines they ex-

press. These factors will be determined by the context in which DCs are stimulated to mature. Bacterial and viral products such as LPS and double-stranded (ds)RNA recruit large numbers of DCs and activate them to a high stimulatory status, whereas endogenous inflammatory cytokines are much less effective (22-24). CD40L and TNF-related activation-induced cytokine (TRANCE) represent additional endogenous stimuli by which T cells can enhance DC stimulatory capacity (25-27) and viability. According to the second DC activation hypothesis, tolerance or priming will be determined by how far pathogens and/or memory T cells raise the activation state of maturing DCs, i.e., high activation state and high numbers will favor priming, whereas low activation and low numbers will induce tolerance or ignorance (Fig. 1). The regulatory role of pathogens, antigen dose, and kinetics of presentation has been amply documented (28). There is now growing evidence that most of this regulation is taking place primarily at the level of DCs.

A Role for DCs in Chronic Inflammation. A long-standing observation has been that tissues undergoing chronic inflammatory reactions contain infiltrates of lymphocytes that are organized in lymph node-like structures. TNF- α and lymphotoxin (LT) are required for this lymphoid neogenesis (29), and recent data suggest that DCs might also play a role in maintaining chronic inflammation via de novo formation of local lymphoid tissue. Ludewig et al. showed that repetitive immunization with DCs carrying a diabetogenic peptide induced chronic inflammation and lymphoid neogenesis, demonstrating that DCs presenting self-antigens are not only potent inducers of autoreactive T cells, but also help to maintain a peripheral immune re-

sponse locally (30). It will be important to consider this as a potential hazard in DC-based antitumor therapies.

Based on the central role played by chemokines, it is tempting to suggest that DCs that mature in inflamed tissues and fail to migrate to the lymph nodes may act as nucleation sites to organize a lymphoid structure that sustains the chronic inflammatory reaction (Fig. 1). Indeed, mature DCs produce constitutive chemokines such as ELC, TARC, and MDC which attract recently activated T cells that have upregulated CCR7 and CCR4 as well as maturing DCs that may sustain the process of lymphoid neogenesis. This mechanism may explain why sites of chronic inflammation such as the rheumatoid arthritis synovia can act as sinks for recently activated T cells such as those that are chronically activated by endogenous antigens, for instance EBV (31). Although these cells are not autoreactive, they may nonetheless aggravate the chronic inflammatory process. For example, they could be activated in an antigen-independent fashion by certain cytokine combinations present in the microenvironment (32), and in this way may provide to DC survival and activation signals (via CD40L and TRANCE) that may be important to sustain the inflammatory process.

As our understanding of DC physiology improves, we realize that we cannot look at these cells simply as the "good guys." Because of their fundamental role in initiating T cell responses, it is perhaps not surprising that they are involved in many aspects of immune regulation, including tolerance and autoimmunity. Understanding the control of DC traffic and activation will provide new avenues for therapeutic intervention.

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Application Number 09/804,584

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thanks!

Interaction of Dendritic Cells with Skin Endothelium: A New Perspective on Immunosurveillance

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Summary

The goal of this study was to determine the mechanisms by which dendritic cells (DCs) in blood could interact with endothelium, a prerequisite to extravasation into tissues. Our results indicate that DCs express both HECA-452-reactive and nonreactive isoforms of P-selectin glycoprotein ligand 1 (PSGL-1) and can tether and roll efficiently on E- and P-selectin under flow conditions *in vitro*. Freshly isolated blood DCs were further observed to roll continuously along noninflamed murine dermal endothelium *in vivo*. This interaction is strictly dependent on endothelial selectins, as shown by experiments with blocking antibodies and with E- and P-selectin-deficient mice. We hypothesize that DCs in blood are constitutively poised at the interface of blood and skin, ready to extravasate upon induction of inflammation, and we showed that cutaneous inflammation results in a rapid recruitment of DCs from the blood to tissues. We propose that this is an important and previously unappreciated element of immunosurveillance.

Key words: inflammation • immunosurveillance • selectins • rolling • extravasation

In the years since their initial description, the central role of dendritic cells (DCs)¹ in the development of acquired immune responses has become widely accepted (1, 2). Tissue DCs, particularly those in tissues which constitute epithelial interfaces with the environment, capture antigens and migrate to lymphoid organs, where they present antigens to T cells (1, 2). Another population of DCs also exists in peripheral blood, where they represent ~0.5% of the circulating PBMCs (3). Extensive study of blood DCs has been limited by their paucity. This population expresses HLA-DR and lacks specific lineage markers or surface activation molecules found on other leukocytes. Although they lack the typical dendritic morphology of mature DCs, they can rapidly acquire these features in culture (3, 4).

A physiological role for blood DCs is unclear, but at least some of these cells are hypothesized to be en route to tis-

sues (3, 4). As such, they must be capable of initiating interactions with vascular endothelium while moving in the blood flow. However, this hypothesis has never been directly tested and the mechanisms regulating this putative process are undetermined (3). The extravasation of leukocytes across endothelia has been described as a multistep cascade of discrete events (5, 6). The initial adhesive step, which involves binding in shear flow, has been associated with a limited subset of surface molecules, including the selectins and the $\alpha 4$ integrins and their respective ligands. In this study, we examine the capacity of blood DCs to complete the initial step involved in leukocyte homing by tethering and rolling under flow, both *in vitro* and *in vivo* along the skin vessel surface. We further test the capacity of DCs to extravasate *in vivo* in the setting of inflammation.

Materials and Methods

Isolation of Fresh Blood DCs. PBMCs were isolated by density gradient separation from blood cells collected from normal donors during plateletpheresis. Fresh blood DCs were isolated from

¹Abbreviations used in this paper: CLA, cutaneous lymphocytic-associated antigen; DC, dendritic cell; PSGL-1, P-selectin glycoprotein ligand 1; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late antigen 4.

PBMCs as described previously (7) or using a commercial blood DC isolation kit (Miltenyi Biotech). Both methods resulted in very pure populations of DCs (HLA-DR⁺ TCR- α/β^+ , CD14⁺, CD56⁻, and CD19⁻; see Fig. 1). Viability was >95% by trypan blue exclusion. Fresh blood DCs isolated by either method were used within 4 h of purification.

Isolation and Culture of Human CD34⁺ Hematopoietic Progenitor Cells. When our experiments required quantities of DCs beyond our means to isolate directly from blood (i.e., for the molecular characterization of selectin ligands expressed on DCs), we used immature cultured DCs, derived from cord blood progenitors (8). CD34⁺ progenitors were isolated from the mononuclear fraction of heparinized umbilical cord blood samples by magnetic separation (CD34⁺ separation kit; Miltenyi Biotech). Cell preparations were routinely >90% CD34⁺ by FACS[®] with viability approaching 100%. Cultures of CD34⁺ progenitors were established by seeding at 10⁵ cells/ml in RPMI 1640 (GIBCO BRL) supplemented with 10% fetal bovine serum, recombinant human (rh)GM-CSF (1,000 U/ml; Immunex Corp.), IL-4/TNF- α (50 U/ml each, R & D Systems), Flt3 ligand (100 ng/ml; Immunex Corp. and R & D Systems), 10 mM Hepes, 2 mM L-glutamine, 5 \times 10⁻⁵ M 2-ME, penicillin (100 U/ml), and streptomycin (100 μ g/ml). Clusters of nonadherent cells with dendritic morphology appeared after 4–5 d of culture and increased in number and size in the following days. We routinely observed a 10–150-fold increase in total cell number after 2–3 wk of culture. Cells were used on days 10–14 when cultures contained cells with dendritic phenotypic characteristics and surface markers (72–85% HLA-DR⁺, 30–80% CD1a⁺, 40–50% CD80⁺, 30–40% CD83⁺, >95% CD14⁺).

Preparation of Murine Bone Marrow-derived DCs. Bone marrow-derived DCs were prepared as published previously (9). In brief, bone marrow cells from FVB mice were depleted of red cells by lysis in ACK lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.3), and the cultures were established in RPMI 1640 (GIBCO BRL) supplemented with 10% fetal bovine serum with recombinant murine (rm)GM-CSF (400 U/ml) and rmIL-4 (150 U/ml; PharMingen). DCs were used between 4 and 6 d after bone marrow harvest. Flow cytometry was performed immediately before the homing experiments and showed immature phenotype DCs expressing class II I-A antigen and moderate amounts of CD40 and B7-2.

FACS[®] Analysis. mAbs used included HECA-452 (PharMingen), anti-human P-selectin glycoprotein ligand 1 (PSGL-1) (PSL-275 and 4H10; gifts from the Genetics Institute, Cambridge, MA), anti-CD14, anti-CD19, anti-CD56, and anti-TCR- α/β (Coulter Corp.), anti-mouse I-A, anti-CD40, and anti-B7-2 (PharMingen). Nonbinding isotype-matched antibodies were used as control reagents. Flow cytometry was performed on a FACScan[®] IV (Becton Dickinson) using CellQuest software (version 1.2). Results are representative of multiple independent experiments.

Immunoblotting. HECA-immunoreactive DCs were positively selected from CD34⁺-derived DCs after 9–10 d of culture using magnetic microbeads (Miltenyi Biotech) as published previously (10). Methods for preparing cell lysates from cultured DCs and for immunoblotting under normal and enhanced reducing conditions were as described previously (10) with the following modifications: the anti-PSGL-1 mAb PL1 (mIgG1; Immunotech) was used at a concentration of 2 μ g/ml. HECA-452 (a gift from Dr. L. Picker, University of Texas/Southwestern, Dallas, TX [11]) was used at 1.2 μ g/ml (rather than 2 μ g/ml). Immunoblots were prepared using 300 μ g of HECA-immunoreactive DC lysate protein per lane for HECA-452 blots and 600 μ g of unse-

lected DCs per lane for PSGL-1 blots. Enhanced reduction of cell lysates was carried out for 5–8 d, rather than 3 d, before SDS-PAGE.

In Vitro Flow Analysis. For E- and P-selectin binding analysis, cells were analyzed in a parallel plate flow chamber using protein A-bound E- or P-selectin IgG chimeras as described previously (12). Washed cells were resuspended in medium with 2 mM Ca²⁺ immediately before use, perfused into the chamber, and allowed to interact under static conditions for 3 min. Flow was initiated at a wall shear stress of 1 dyn/cm², and interactions were observed for an additional 1 min. Shear was subsequently increased in a stepwise fashion every 10–30 s, and the percentage of rolling cells remaining bound was determined at each step. Non-specific binding was defined as cells remaining bound in 5 mM EDTA perfused at 50 dyn/cm². The percentage of cells able to form adhesions was calculated as the number of cells remaining bound at 1 dyn/cm² after a 3-min static incubation, divided by the number of cells present just before reinitiating flow. The number of cells attached/ μ m²/min was determined by counting the number of new tethers observed in the field during 1 min of flow at 1 dyn/cm².

Intravital Microscopy. Normal adult Swiss Webster mice, mice deficient in both E- and P-selectin (E/P-selectin^{-/-}; reference 13), and control wild-type mice from the same strain as the deficient mice (C57BL/129SV) were anesthetized by intraperitoneal injection of saline (10 ml/kg) containing 5 mg/ml ketamine and 1 mg/ml xylazine. The hair on the left ear as well as on the submandibular area of the neck was removed using hair removal lotion. A PE-10 polyethylene catheter was inserted into the right common carotid artery of a thermo-controlled mouse whose left ear was covered with glycerol and gently positioned between a microscope slide and a coverslip under an intravital microscope (model IV-500; Mikron Instruments). DCs were fluorescently labeled (2.5 μ g calcein/ml/10⁷ cells; Molecular Probes, Inc.) and introduced by retrograde injection into the right carotid artery. Fluorescent DCs in ear microvessels were visualized by stroboscopic fluorescent epi-illumination using infinity-corrected water-immersion optics (Carl Zeiss) and a silicon-intensified target camera (Dage). Rolling fractions were determined as the percentage of interacting cells in the total flux of fluorescent DCs that passed through each venule during the same period. The velocities of individual rolling cells were determined by off-line analysis of videotapes using a PC-based interactive image analysis system (14). Hemodynamic parameters were determined in 3 animals, 4 venules, and 82 noninteracting cells (12, 12, 28, and 30 fast cells per venule) for cultured DCs in wild-type mice; in 3 mice, 3 venules, and 96 cells (31, 32, and 33 per venule) for cultured DCs in E/P-selectin^{-/-} mice; and in 2 wild-type mice, 3 venules, and 55 cells (18, 18, and 19 per venule) for freshly isolated DCs. Anti-mouse E- and P-selectin blocking antibodies, 9A9 and 5H1, respectively (from B. Wolitzky, Hoffman-La Roche, Inc., Nutley, NJ), were used at 100 μ g/mouse and injected into the blood circulation 5 min before the infusion of the cells.

Homing of DCs during a Hypersensitivity Contact Reaction in Mice. FVB mice were sensitized with a classical contact sensitizer, oxazolone (100 μ l of 2% oxazolone in acetone on abdominal skin) 6 d before challenge with 10 μ l of 0.8% oxazolone on both sides of the right ear. The left ear was not treated and is referred to as the control ear. Immature bone marrow-derived DCs were labeled with ⁵¹Cr (NEN) as follows: 10⁷ DCs/ml were resuspended in RPMI containing 20% FCS and incubated for 1 h with 200–400 μ l of [⁵¹Cr] sodium chromate (1 mCi/ml) at 37°C in 5% CO₂. The cells were washed twice in RPMI, and 5 \times 10⁶

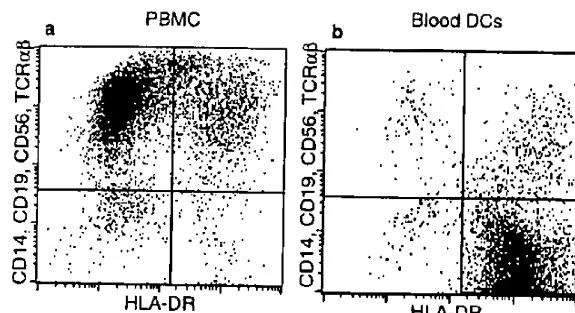


Figure 1. Blood DCs express HLA-DR but do not express specific lineage markers CD14, CD19, or CD56 or any TCR (reference 3). (a) They represent <1% of the PBMCs (reference 3). After immunomagnetic depletion of T cells, monocytes, and NK cells, using CD3, CD11b, and CD16 antibodies and positive selection of CD4⁺ leukocytes (blood DC isolation kit from Miltenyi Biotec), a population of >90% pure blood DCs is obtained (b).

DCs/mouse were infused into the tail vein 48 h after the challenge was applied. The mean specific radioactivity of injected DCs was 288 cpm/10³ cells (range 88–620). 6 h after DC infusion, the mice were killed, and the radioactivities of both ears were counted in a gamma counter.

Calcein-labeled DCs were also used in similar experiments. 100 µg of PE-conjugated CD31 mAb (PharMingen) was infused into the tail veins of mice 5 min before they were killed in order

to visualize the vessels by confocal microscopy. Ears were split in two halves parallel to their broad surfaces using fine forceps, put between a microscope slide and a coverslip, and examined using a BioRad MRC-1024 confocal imaging system.

Results

DCs Uniformly Express the Selectin Ligand PSGL-1/Cutaneous Lymphocyte-associated Antigen. Magnetic separation of blood DCs allowed the recovery of >85% pure DCs, as defined by the expression of HLA-DR and the lack of specific lineage markers CD14, CD56, CD19, and TCR- α/β (Fig. 1). We examined DC expression of three leukocyte cell surface molecules known to mediate binding under physiologic flow conditions: PSGL-1, L-selectin, and the integrin $\alpha 4\beta 1$. Roughly 50% of freshly isolated DCs expressed L-selectin (not shown), whereas $\alpha 4\beta 1$ (not shown) and PSGL-1 (Fig. 2 a) were expressed by 100% of the cells. We had previously shown that PSGL-1 on T cells could be expressed in an uniquely glycosylated form that reacts with an oligosaccharide-specific antibody called HECA-452 and binds to both E- and P-selectin under flow (10, 11, 15). When expressed on memory T cells, this isoform of PSGL-1 is known as cutaneous lymphocyte-associated antigen (CLA [10]) and is thought to direct the extravasation of T cells into inflamed skin.

Although HECA-452 immunoreactivity has been shown on Langerhans cells, a subset of DCs (16, 17), neither the

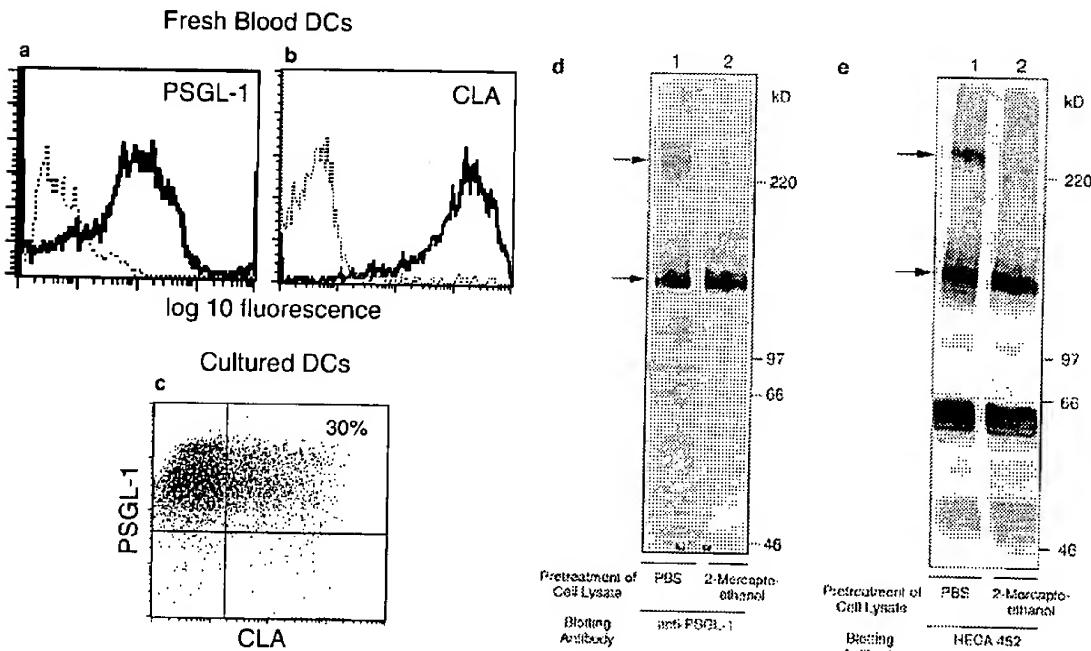


Figure 2. (a and b) Freshly isolated DCs homogeneously express P- and E-selectin ligand, PSGL-1/CLA. FACS[®] analysis of cultured DCs stained with mAb to PSGL-1 (PSL-275) and to CLA (HECA-452). (c) Cultured DCs express PSGL-1 (4H10), and 30% (30–50%) of them also express CLA. (d and e) Immunoblotting of cultured DC lysates with anti-PSGL-1 (d) and HECA-452 (e) shows that the CLA epitope is expressed on a single glycoprotein. Analysis under standard reducing conditions (lane 1) revealed specific immunoreactive bands at 240 kD (dimer) and 140 kD (monomer) with each specific antibody; and under enhanced reducing conditions (lane 2), only one specific band at 140 kD (monomer) was seen for both HECA-452 and anti-PSGL-1 immunoreactivities. The bands at 50 and 60 kD in the HECA-452 blot represent the HECA antibody used for immunomagnetic separation, as shown previously (reference 10).

structure of the molecules carrying this oligosaccharide epitope nor its functional capability as a selectin ligand on DCs has been reported. FACS® analysis was performed using 4H10 or PSL-275, two mAbs that recognize peptide determinants in the P-selectin binding domain of PSGL-1, and HECA-452, an IgM mAb that recognizes a carbohydrate epitope similar to sialyl Lewis X (18) and defines CLA expression on T cells (11). PSGL-1 and CLA were expressed by virtually all freshly isolated blood DCs (Fig. 2, a and b). PSGL-1 was also homogeneously expressed on ex vivo-derived DCs (cultured for 10–14 d), whereas HECA-452 was expressed on only a subset of these cells (25–40%; Fig. 2 c). To further characterize the structure of these molecules on DCs, immunoblotting of cell lysates with HECA-452 and anti-PSGL-1 antibodies was performed (Fig. 2, d and e). Both antibodies recognized bands of ~240 and 140 kD under normal reducing conditions, representing the dimeric and monomeric forms of CLA/PSGL-1 as described previously (10). Enhanced reduction resulted in conversion of all immunoreactivity to a single band of 140 kD, indicating that in DCs, as in T cells, the epitope recognized by HECA-452 is expressed on only a single major surface glycoprotein, PSGL-1.

DCs Tether and Roll on Both E- and P-selectin In Vitro. Having identified a candidate ligand for E- and P-selectin,

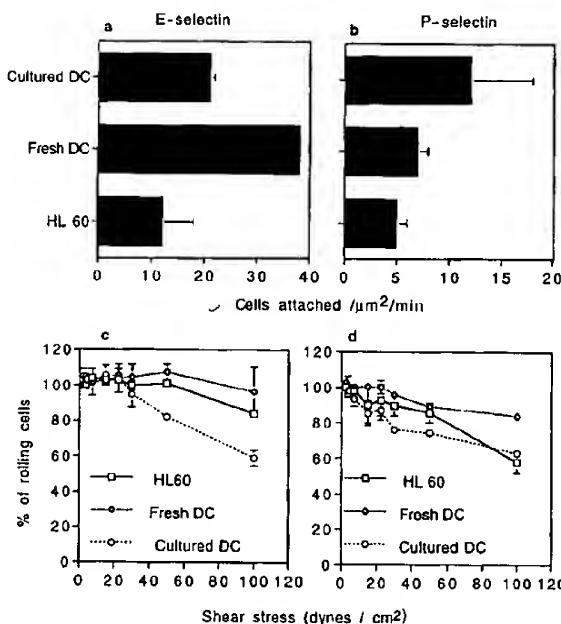


Figure 3. DCs tether and roll on E- and P-selectin in vitro. Interaction of DCs with E- and P-selectin was assessed in a parallel plate flow chamber. HL60 cells are included as a positive binding control. Both fresh and cultured DCs formed tethers on E-selectin (a) and P-selectin (b) at 1 dyne/cm² and rolled in a calcium-dependent fashion. DCs bound to either E- (c) or P-selectin (d) showed strong resistance to detachment by increasing wall shear stress with >80–100% of cells remaining bound at 25 dyne/cm². E- and P-selectin binding was abolished by perfusion of medium containing 5 mM EDTA. The data presented are the mean and range of two experiments and are representative of several independent observations.

the ability of these cells to tether and roll on both E- and P-selectin was assessed in vitro using a parallel plate flow chamber (19). HL60 cells, a promyelocytic cell line known to bind to E- and P-selectin (20), were included as a control. DCs bound to both E-selectin and P-selectin with high affinity and rolled in shear flow (Fig. 3, a and b). The percentage of cells interacting with E- or P-selectin after 3 min of static incubation varied between 77 and 94%. Resistance to detachment from E- and P-selectin during step-wise increases in shear flow was extremely high (Fig. 3, c and d). Even at a wall shear stress as high as 100 dyne/cm², >50% of the tethered cells remained bound and rolling.

No significant differences were observed between fresh and cultured DCs within the physiologic shear range of postcapillary venular blood flow (1–25 dyne/cm²). However, at high shear stresses (50–100 dyne/cm²) fresh DCs showed an increased shear resistance compared with cultured DCs (Fig. 3, c and d).

DCs Tether and Roll on Endothelium of Noninflamed Skin. While the parallel plate flow chamber assay is a useful model for assessment of distinct adhesion pathways under defined shear flow conditions, the advent of intravital microscopy has made possible the direct observation of leukocyte interaction with postcapillary venular endothelium in vivo (14). The ability of human E- and P-selectin ligands to interact effectively with murine selectins allows human leukocytes to be studied in mouse microvessels (Stein, J., and U.H. von Andrian, unpublished data). Selectin-dependent rolling of leukocytes has been observed in mouse skin microcirculation without surgical manipulation of the tissue, thus allowing observation of basal interactions between leukocytes and noninflamed endothelium (21; and Ulfman, L.H., and U.H. von Andrian, manuscript submitted for publication). We tested the hypothesis that the high level of CLA/PSGL-1 expression on blood DCs would permit tethering and rolling of these cells in uninflamed ear postcapillary venules. Fluorescently labeled human DCs were injected through the right common carotid artery into the aortic arch of anesthetized mice and observed directly in the microcirculation of the left ear. In the absence of any overt skin inflammation, a significant fraction of injected blood DCs were observed to tether and roll in cutaneous microvessels in vivo (Table I). Injected cells interacted exclusively with postcapillary venules, where the extravasation of other leukocytes is known to take place (22), and not with arterioles or capillaries (Fig. 4 a). A significant proportion of cultured DCs also rolled in murine ear postcapillary venules, and displayed comparable rolling properties (Table I).

Interaction of DCs with Endothelium In Vivo Is Mediated by Endothelial Selectins. To assess the role of endothelial selectins in DC binding to postcapillary venules, we performed experiments in the presence of blocking antibodies as well as in E/P-selectin^{-/-} animals (13). As shown in Table I, pretreatment with anti-E- and P-selectin mAbs effectively blocked interaction of DCs with skin vessels. Objective assessment of leukocyte endothelial interaction was obtained by comparing the velocity distribution of cells ob-

Table I. DC Rolling Parameters in Ear Venules

	Fresh DCs		Cultured DCs	
	No Antibody	Plus antibodies*	Wild-type mice	E/P-selectin ^{-/-} mice
No. of animals/venules/cells	3/31/534	2/14/241	5/27/627	3/31/96
Rolling fraction (%)	26.5 ± 7.5	0.01 ± 0	50 ± 12.4	0.3 ± 0
Mean rolling velocity (μm/s)	59.8 ± 35.4	—	57.8 ± 4	—
Mean blood flow velocity (μm/s)	1,549 ± 633	ND	1,294 ± 264	1,434 ± 563
Wall shear rate (s ⁻¹)	406 ± 228	ND	339 ± 78	253 ± 39
Shear stress (dyn/cm ²)	10.1 ± 5.7	ND	8.5 ± 2	6.3 ± 1

Rolling fractions were obtained from the numbers of cells and venules indicated in the table. Hemodynamic parameters were determined in 2 animals, 3 venules, and 55 noninteracting cells for freshly isolated DCs; 4 animals, 4 venules, and 82 noninteracting cells for cultured DCs in wild-type mice; and in 2 animals, 2 venules, and 96 noninteracting cells for the E/P-selectin^{-/-} mice and were calculated as described previously (reference 22). Centerline blood flow velocity (V_{CL}) was calculated from the velocity of the fastest cell traversing the field during the period of observation. The mean blood flow velocity was estimated assuming a parabolic flow profile as $V_{blood} = V_{CL}/1.6$. The wall shear stress was approximated from the wall shear rate assuming the viscosity of blood to be 0.025 poise.

*100 μg of anti-mouse E- and P-selectin blocking antibodies (9A9 and 5H1, respectively; provided by B. Wolitzky) was injected per mouse.

served in comparable size vessels in E/P-selectin^{-/-} and control mice (Fig. 4 b). Cells traveling below V_{crit} , the calculated velocity of noninteracting cells at the endothelial surface of a blood vessel with a parabolic flow profile (22),

are defined as interacting with (i.e., rolling on) the vessel walls while the cells moving above V_{crit} are traveling free in the bloodstream. Although ~50% of labeled DCs observed in the vessels of wild-type mice formed rolling interactions,

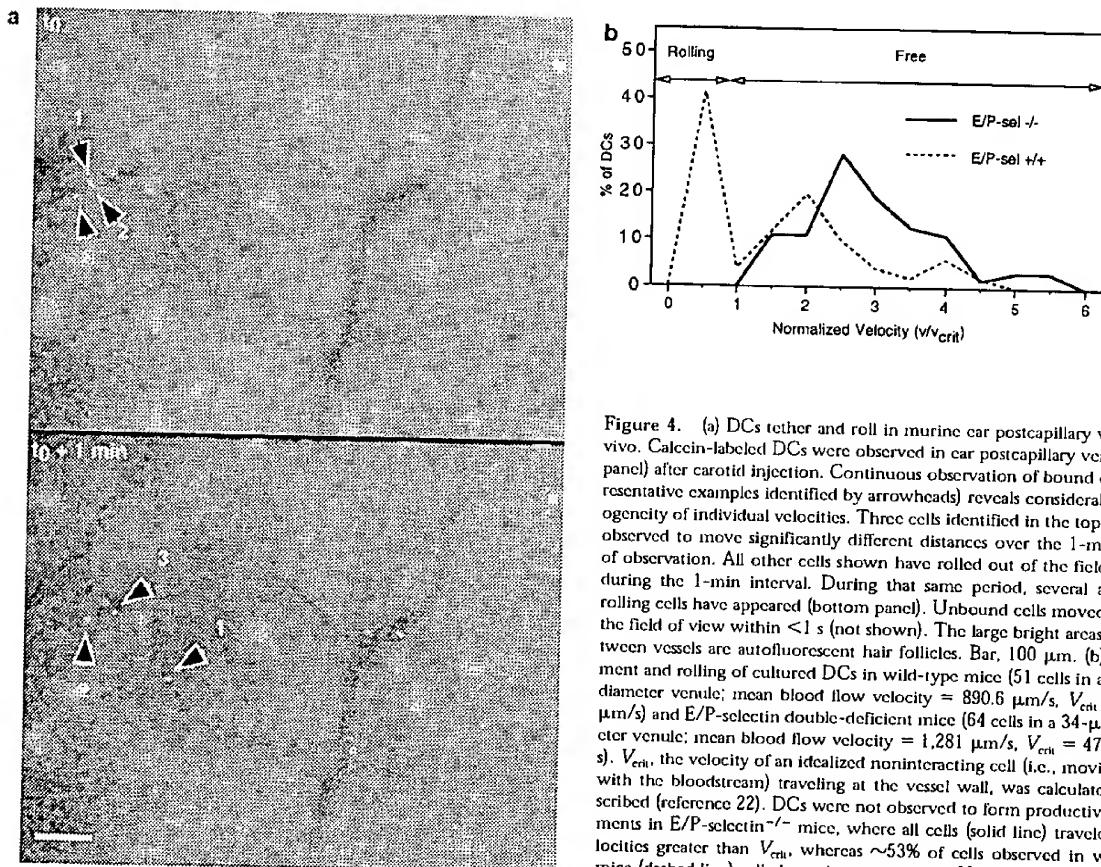


Figure 4. (a) DCs tether and roll in murine ear postcapillary venules in vivo. Calcine-labeled DCs were observed in ear postcapillary venules (top panel) after carotid injection. Continuous observation of bound cells (representative examples identified by arrowheads) reveals considerable heterogeneity of individual velocities. Three cells identified in the top panel are observed to move significantly different distances over the 1-min period of observation. All other cells shown have rolled out of the field of view during the 1-min interval. During that same period, several additional rolling cells have appeared (bottom panel). Unbound cells moved through the field of view within <1 s (not shown). The large bright areas seen between vessels are autofluorescent hair follicles. Bar, 100 μm. (b) Attachment and rolling of cultured DCs in wild-type mice (51 cells in a 28-μm-diameter venule; mean blood flow velocity = 890.6 μm/s, $V_{crit} = 388.2$ μm/s) and E/P-selectin double-deficient mice (64 cells in a 34-μm-diameter venule; mean blood flow velocity = 1,281 μm/s, $V_{crit} = 471.7$ μm/s). V_{crit} , the velocity of an idealized noninteracting cell (i.e., moving freely with the bloodstream) traveling at the vessel wall, was calculated as described (reference 22). DCs were not observed to form productive attachments in E/P-selectin^{-/-} mice, where all cells (solid line) traveled at velocities greater than V_{crit} , whereas ~53% of cells observed in wild-type mice (dashed line) rolled at velocities less than V_{crit} .

no significant interactions were observed in endothelial selectin-deficient animals (<0.5% labeled cells traveling below V_{crit}). Besides E- and P-selectin ligands, the only known leukocyte adhesion molecules capable of mediating tethering and rolling on vascular endothelium are L-selectin, very late antigen 4 (VLA-4) and $\alpha 4\beta 7$. The vascular ligands for L-selectin and integrin $\alpha 4\beta 7$, peripheral lymph node addressin (PNAd) and mucosal addressin cell adhesion molecule 1 (MAdCAM-1), respectively, are not expressed in normal skin endothelium (23, 24) and therefore would not be expected to participate in these interactions. However, murine vascular cell adhesion molecule 1 (VCAM-1) may be present, and can readily bind human VLA-4 (integrin $\alpha 4\beta 1$ [25]). The lack of interactions observed in anti-selectin antibody-blocked or selectin-deficient animals unambiguously indicates that the spontaneous tethering and rolling of DCs observed in wild-type animals is mediated by one or both of the endothelial selectins constitutively expressed on skin postcapillary venules, and that a major role for other known ligand pairs can be excluded, at least in the dermal vascular bed studied here. We cannot rule out unknown or uncharacterized ligand pairs that do not bind across species. However, these data are consistent with observations in rats, mice, and rabbits that the constitutive rolling of endogenous leukocytes in skin venules is mediated primarily via interaction with vascular selectins (26, 27; and Ulfman, L.H., and U.H. von Andrian, manuscript submitted for publication), and not via $\alpha 4$ integrins, or L-selectin, in contrast to other anatomic sites such as bone marrow (28) or peripheral lymph nodes (29).

DCs Are Recruited into Inflamed Skin. Endothelial selectins and other adhesion molecules involved in leukocyte recruitment are upregulated during cutaneous inflammation (30). Since blood DCs express functional selectin ligands, chemokine receptors, and cell surface integrins (2, 3, 7), they should have all the requisite molecules necessary for recruitment into inflamed tissues. To test whether DCs could be recruited to inflamed skin, we measured accumulation of radioactively labeled DCs in ear skin of oxazolone-sensitized mice challenged with antigen on one ear 48 h before the infusion of the cells. To avoid potential incompatibility between human and mouse elements in the downstream portions of the adhesion/extravasation cascade, we used immature murine bone marrow-derived DCs, grown for 4–6 d with GM-CSF and IL-4 (9). ^{51}Cr -labeled DCs (mean specific activity = 288 cpm/10³) were injected intravenously, and the mice were killed 6 h later. Radioactivity counts were significantly higher in inflamed ears compared with control ears (cpm ratio in challenged versus control ears ranged from 1.7 to 9; mean ratio = 3.5, $P < 0.01$; Fig. 5, a and b). Cells recovered in inflamed ears ranged from 0.02 to 0.25% of injected cells or 10³ to 1.25 \times 10⁴ cells/ear. This is consistent with previous reports of cells recovered from significantly larger areas of inflamed skin samples after intravenous injection of radiolabeled T cells (31, 32). A trivial explanation would be that this increase in cpm resulted from an increase of the volume of blood in the inflamed ear, and not from extravasation of

DCs. One argument against this explanation is that this phenomenon required living cells, since no specific label was found in the skin after injection of dead DCs (not shown). To confirm that this increase in radioactivity actually represented extravasated DCs, we performed similar experiments with calcein-labeled DCs and injected PE-conjugated anti-mouse CD31 mAb 5 min before the animals were killed, to stain the vessels in red. Confocal microscopy of inflamed ears showed extravascular DCs (green) clearly outside of the skin vessels and in the extravascular tissue (Fig. 5, c–e), confirming the actual extravasation of the DCs, whereas no extravascular cells were observed in noninflamed ears. It should be emphasized that this experiment was designed to be purely qualitative and to address the anatomical location rather than absolute number of extravasated cells.

Discussion

In this study, we have shown that DCs efficiently tether and roll on E- and P-selectin in vitro. Taking advantage of previous studies showing that relevant adhesion molecules which mediate leukocyte rolling (i.e., selectins/selectin ligands and VLA-4/VCAM-1) are functional across human and murine species (25; and Stein, J., and U.H. von Andrian, unpublished data), we have demonstrated a direct interaction between human DCs and murine skin postcapillary venules in vivo. This confirms the hypothesis that DCs can participate in the essential first step of the adhesion/extravasation pathway on vessels expressing E- and/or P-selectin. This interaction takes place in the absence of overt inflammation and may also facilitate the extravasation of DCs into injured skin. To undergo extravasation, rolling cells have to be activated (e.g., by chemoattractants) and to bind via surface integrins to vascular cell adhesion molecules (e.g., intercellular adhesion molecule 1 [ICAM-1] and VCAM-1). DCs express multiple chemokine receptors (7, 33, 34). As described previously for different types of lymphocytes (35), it was recently shown that DCs at different maturational stages express patterns of chemokine receptors that seem to correlate with recruitment to distinct anatomic sites (36–38). DCs also express the cell surface integrins LFA-1 and VLA-4 (2, 3), the ligands for ICAM-1 and VCAM-1, respectively. Thus, DCs appear to have all the surface molecules required to participate in the adhesion/extravasation cascade. We have confirmed this potential by demonstrating active recruitment of immature cultured DCs into inflamed skin after intravenous injection and by direct visualization of extravasated DCs using confocal microscopy. These experiments were highly reproducible despite the low absolute number of cells found in ear skin. The association between skin homing potential and in vivo rolling on murine skin venules is strengthened by the observation that naive lymphocytes, which do not express PSGL-1/CLA and do not normally migrate to skin (15), do not show significant interaction with murine skin endothelium in vivo. In contrast, T cells isolated from a cutaneous T cell lymphoma patient, which express PSGL-1/

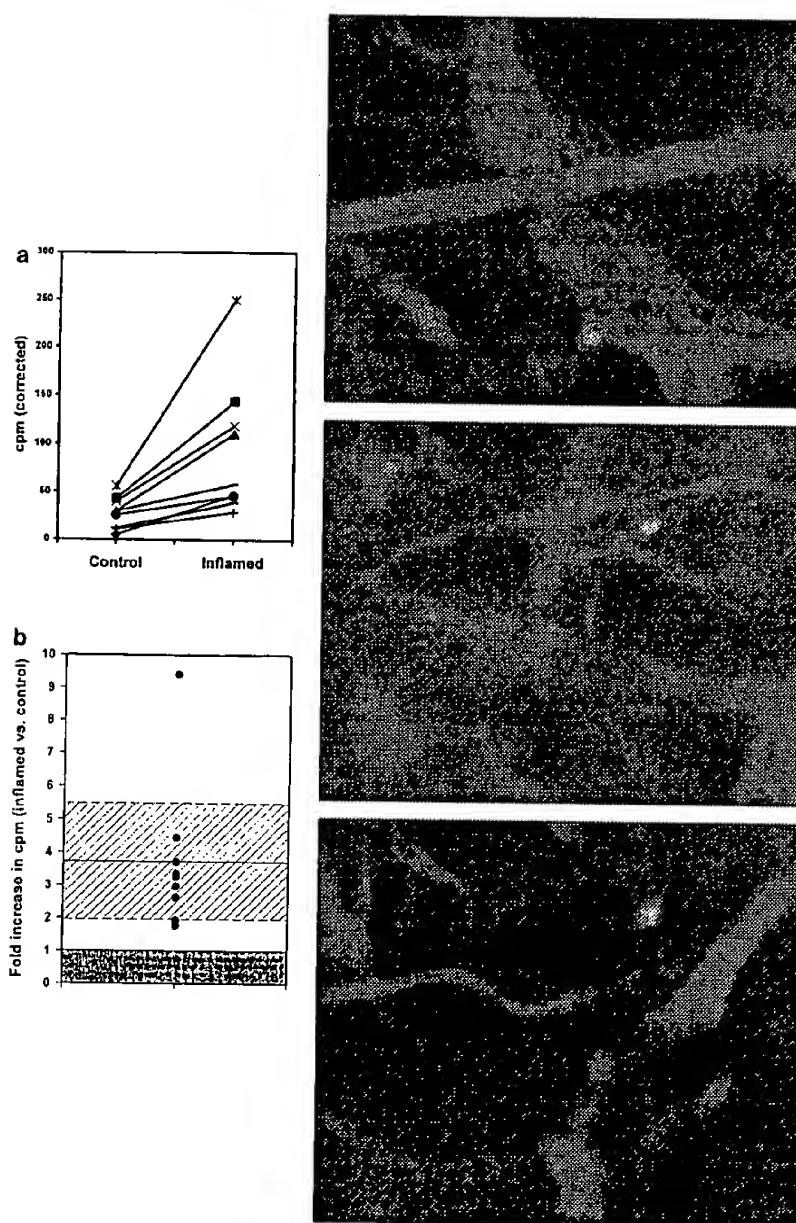


Figure 5. Immature murine bone marrow-derived DCs are acutely recruited into inflamed skin. Mice sensitized to oxazolone were challenged on the right ear 48 h before intravenous infusion of 5×10^6 ^{51}Cr -labeled DCs. 6 h after DC infusion, the mice were killed and the cpm were compared in both ears. (a) Results obtained in nine mice are shown; cpm values are corrected by subtraction of the background radioactivity. There is a significant difference between the challenged and control ear ($P < 0.01$, Student's *t* test). (b) Shows the fold increase in cpm measured in inflamed ears versus contralateral control ears for nine independent determinations. The area from 0 to 1 is shaded. Values above the shaded area reflect an increased number of labeled cells in the inflamed ear, and values within the shaded area reflect a decreased number of cells in the inflamed ear relative to control. The solid line and hatched field represent the mean and 95% confidence interval for fold increase of inflamed ear counts versus control ($P < 0.01$, Student's *t* test). (c–e) Confocal microscopy images of an inflamed ear 6 h after infusion of 20×10^6 calcine-labeled murine bone marrow-derived DCs. 100 μg of PE-conjugated mAb anti-mouse CD31 (PharMingen) was infused 5 min before the animal was killed, in order to visualize the vessels. Several fields of the same ear (photographed through a 40 \times objective) are shown: (c) a calcine-labeled DC is seen just outside of the vessel wall; (d) another DC is seen between two small vessels; and (e) a calcine-labeled DC has clearly extravasated and is shown deeper in the surrounding tissues. No extravascular cells were observed in contralateral, noninflamed ear.

CLA at high levels and do target to skin, roll spontaneously on uninflamed mouse ear postcapillary venules (Robert, C., unpublished data).

There are at least two non-mutually exclusive reasons why blood DCs should be able to migrate to peripheral tissues. First, they may represent a pool of tissue DC precursors that continuously extravasate to repopulate tissues with resident DCs (e.g., for skin, the dermal DCs and/or the Langerhans cells). Second, blood DCs may also represent a circulating pool of APCs that are acutely recruited to sites of inflammation. Consistent with this hypothesis, it has been reported that the number of DCs is increased in tis-

sues in different models of inflammation (39–41), though DCs have never been shown to be actively recruited from the blood.

We showed that blood DCs constitutively interact with normal murine skin endothelium *in vivo* via selectins. This spontaneous rolling of CLA⁺ DCs in skin microvessels, in the absence of an inflammatory stimulus, suggests that a large number of blood DCs may be interacting with cutaneous postcapillary venules at any given time. This continuous interaction is likely to play a role in the seeding of peripheral tissues with DCs to provide the tissue-resident pool of APCs. We also showed that DCs can be acutely re-

cruted into inflamed skin. We propose that constitutive selectin-mediated rolling represents surveillance of the luminal aspect of skin endothelium by blood DCs for activating signals (e.g., chemokines). This enhances the ability of these potent APCs to rapidly extravasate when they encounter inflamed endothelium, as in the setting of skin injury or infection. Rapid recruitment of blood DCs, facilitated by these constitutive reversible interactions between blood DCs and endothelial surfaces, could enhance local antigen capture and antigen presentation activity both at the site of inflammation and in the draining lymph nodes. Teleologically, this may represent a previously unrecognized element of skin immunosurveillance and a highly adaptive interface between innate and acquired immunity.

It is interesting to note that DCs are often referred to as "nature's adjuvant" (1). The rapid recruitment of APCs to sites of inflammation may be an important element in determining the efficiency of primary immune responses, as seen, for example, in the enhancing effect of adjuvant-induced inflammation on the response to immunization.

The relative importance of rapid recruitment of DCs and monocytes in this process is at present unknown. We favor the hypothesis that DCs provide an immediate bolus of APCs that can present antigens to CLA⁺ memory T cells that have extravasated under similar conditions in inflamed skin. Monocytes also express PSGL-1/CLA and can tether and roll on E- and P-selectin in vitro (Kieffer, J.D., unpublished data). They can differentiate into DCs in vitro (42) but require at least 48 h to do so, and therefore may be more important in the amplification and perpetuation of the immune response. These hypotheses are currently being tested experimentally.

In summary, we show that immature blood DCs can participate in the leukocyte adhesion/extravasation pathway and be acutely recruited to inflamed skin. Constitutive interactions between DCs and normal skin endothelium may represent an important immunosurveillance mechanism by which DCs could sample local activating signals and rapidly extravasate into inflamed tissues.

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thanks!

Mechanisms of Tolerance Induction in Major Histocompatibility Complex Class II-restricted T Cells Specific for a Blood-borne Self-Antigen

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Summary

Transgenic mice expressing a major histocompatibility complex class II-restricted T cell receptor with specificity for a natural self-antigen, the fifth component of complement, were generated to analyze the mechanism of tolerance induction to a blood-borne self-protein. In the absence of C5 protein thymocytes from T cell receptor transgenic mice develop into mature CD4 single positive cells which emigrate into the periphery and mount C5-specific T cell responses upon immunization with C5. In the presence of circulating C5 protein, CD4 single positive thymocytes do not develop. Negative selection occurs late in thymic ontogeny leaving the bulk of CD4⁺8⁺ thymocytes unaffected. This phenotype may be due to a delay in contact with self-antigen presentation which, under physiological conditions, is inefficient in the cortex of C5⁺ mice, and therefore does not affect most immature double positive thymocytes. In contrast, in vitro exposure to C5⁻-presenting dendritic cells or in vivo injection of C5 peptide results in deletion of double positive thymocytes. C5⁺ transgenic mice are tolerant in vivo, but contain T cells in spleen and lymph nodes that secrete interleukin 2 and interferon γ in response to C5 activation in vitro. When crossed onto a Rag1^{-/-} background to prevent endogenous T cell receptor rearrangements, these peripheral potentially autoreactive cells do not appear. This indicates that endogenous T cell receptor rearrangements possibly leading to the expression of two receptors might be a prerequisite for their survival and export into the periphery.

Negative selection of potentially self-reactive T cells is important for induction and maintenance of self-tolerance. A wide range of examples shows that tolerance induction in MHC class I-restricted T cells is to a large degree effected by physical deletion of immature CD8⁺ thymocytes (1-3). In addition, nondeletional mechanisms like down-regulation of coreceptors (4), or TCR (5), and induction of anergy (6) prevent activation of potentially self-reactive cells. Tolerance in MHC class II-restricted T cells has likewise been attributed to the deletion of CD4⁺8⁺ thymocytes based on studies in which the cognate peptide was injected into transgenic mice with an ovalbumin-specific, class II-restricted TCR (7). It was unclear how self-tolerance is achieved in the presence of a self-protein that requires internalization, processing, and presentation by MHC class II molecules. To address this question we have generated transgenic mice with a MHC class II-restricted TCR specific for a natural circulating self-protein, the fifth component of complement (C5), and analyzed development of transgenic T cells in the presence or absence of self-antigen. Furthermore, the functional behavior of mature T cells in the periphery of C5⁻ or C5⁺ TCR transgenic mice was analyzed. The findings are that C5⁻ TCR transgenic mice (C5⁻Tg⁺) develop single CD4⁺ T cells with high levels of TCR. Mature cells in the periphery of these mice are activated by C5 protein to secrete IL-2 and

IFN- γ . C5⁺ transgenic mice (C5⁺Tg⁺) do not develop mature CD4⁺ single positive cells, but show only a slight reduction in the number of CD4⁺8⁺ cells in the thymus. Thymic tolerance induction to this blood-borne self-antigen occurs late in development, probably at the transition to single positive cells, which may be due to delayed exposure to self-antigen presentation. Although C5⁺ transgenic mice appear fully tolerant in vivo, their splenic T cells can be activated to secrete IL-2 and IFN- γ in response to C5 in vitro. The presence of anergic, but reactivatable C5-specific T cells is confined to mice that can undergo endogenous rearrangements of alternative TCR genes. C5⁺ transgenic mice bred onto a Rag1^{-/-} background do not have any T cells in the periphery. These data indicate that, similar to class I-restricted T cells, thymic deletion is the major mechanism for tolerance induction in class II-restricted T cells even if the onset of deletion may be delayed due to limitations in self-antigen presentation. The escape from this mechanism appears to be correlated to the capacity to undergo endogenous TCR rearrangements.

Materials and Methods

Animals. CBA/Ca (C5⁺) and A/J (C5⁻) mice are maintained under specific pathogen free conditions at the National Institute

for Medical Research, Mill Hill. The congenic strain A.C5⁺ was kindly provided by Dr. F. Gervais at the Montréal General Hospital Research Institute (Montréal, Canada) (8) and is now bred in Mill Hill. Rag1^{-/-} mice were obtained from Dr. E. Spanopoulou (The Rockefeller University, New York) (9).

Generation of Transgenic Mice. To generate transgenic mice we used the TCR from clone A18 isolated from an A/J mouse immunized with C5 protein (10). Its variable region is encoded by V α 11.1(a)-J α TA37 and V β 8.3-DJ β 2.6. The α chain of the TCR was identified by reverse transcription of total RNA from A18 hybridoma cells and PCR with a panel of primers specific for respective families of V α chains and a C α primer from the constant region (11). The β chain variable region was identified by FACS® (Becton Dickinson & Co., Mountain View, CA) staining with antibodies F23.1 (V β 8.1, 2, 3) (12) which stained positive, and antibodies KJ16 (V β 8.1, 2) (13) and F23.2 (V β 8.2) (14) which both did not stain hybrid A18.

An NcoI-NcoI fragment containing the variable region of the α chain was generated from total RNA by PCR with Pfu polymerase and enzyme digestion. Oligonucleotides used for amplification were the C α primer and the V α 11.1-specific mutational oligonucleotide: 5'-TTG CAG GAC CCA TGG GGA TCA GGT GGA GCA GAGT-3'. Full-length cDNA was reconstituted by ligation with a partial NcoI-NcoI fragment containing leader and constant α chain sequences of F5 TCR in pATX (15, from D. Kioussis, National Institute for Medical Research, London, UK). Similarly, a PCR fragment encoding leader and variable region of the A18 β chain was trimmed with NcoI and C β II and inserted into a C β II-NcoI fragment of the F5 β TCR chain cDNA in pATX. The cDNA constructs were sequenced and BamHI-XbaI (α) and BamHI-EcoRI (β) fragments were inserted into a blunt-ended EcoRI site of the Sall-BamHI fragment of the human CD2 minigene in pBluescript (16). After a BamHI-XbaI segment with the CD2 LCR was added, both α and β constructs were isolated as Sall-NotI and Sall-XbaI fragments, respectively, purified by anion-exchange HPLC on a GenFax column (Waters-Millipore, Milford, MA) and mixed together in equimolar quantities. The mixture was used for microinjections into CBA \times CBA oocytes. Transgenic founders were either maintained on the CBA (C5⁺) background or backcrossed to A/J (C5⁻) or congenic A.C5⁺ mice.

Cell Cultures. For functional tests of C5 reactivity spleens from transgenic mice or nontransgenic controls were subjected to enzyme digestion with a cocktail of collagenase (1.6 mg/ml CLS4; Worthington Biochemical Corp., Freehold, NJ) and DNase (0.1% Fraction IX; Sigma Chemical Co., Poole, UK) for 60 min at 37°C. Cell suspensions were washed twice and plated into 96-well U-shaped microtiter plates (Costar Corp., High Wycombe, UK) at a density of 2 \times 10⁵ cells/well in the presence or absence of C5 or C5 peptide. The culture medium was Iscove's modified Dulbecco medium supplemented with 5% heat inactivated FCS, 2 \times 10⁻³ M L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 5 \times 10⁻⁵ M mercaptoethanol. Supernatant from cultures incubated for 48 h was tested for the presence of IL-2 by its ability to support the growth of the IL-2-dependent cell line, CTLL. Supernatant from cultures incubated for 72 h was tested for IFN- γ activity by a sandwich ELISA as previously described (17). Briefly, antibodies to mouse IFN- γ were used for coating ELISA plates, followed by the addition of supernatants to be tested for the presence of IFN- γ , capture by a second biotinylated IFN- γ -specific antibody, and detection with Streptavidin conjugated with horseradish peroxidase.

Bone Marrow Dendritic Cells as APC. Bone marrow-derived

dendritic cells, generated as described previously (18) were used as APC for the results described in Figs. 2 and 6. The source of GM-CSF was supernatant from hypoxanthine-aminopterin-thymidine-sensitive Ag8653 myeloma cells transfected with murine GM-CSF cDNA which was isolated from a T cell clone by PCR and inserted into the vector BCMGSNeo kindly provided by Dr. H. Karasuyama (Basel Institute for Immunology, Basel, Switzerland) (19). Thymic dendritic cells and macrophages were isolated from C5⁺ mice as previously described (18).

C5 Antigen Preparation and C5 Peptide. C5 was purified from ascites fluid by affinity chromatography as described (20). The C5 epitope recognized by hybrid A18, which donated the transgenic TCR, was identified after digestion of C5 by V8 endoproteinase (Boehringer Mannheim, Mannheim, Germany) and subsequent separation of peptides on a reversed phase HPLC column. A functionally active fraction was sequenced and peptide 107-121 was synthesized by the National Institute of Medical Research (NIMR) peptide synthesis facility.

In Vitro Apoptosis Set Up (22). 2 \times 10⁵ thymocytes per well of a 96-well U-bottomed plate were cultured for 10-12 h with 2 \times 10⁴/well dendritic cells in the presence or absence of C5 protein or peptide. Cells were then harvested and analyzed in FACS®.

In Vivo Depletion of Double Positive Thymocytes. C5⁻Tg⁺ mice received daily intraperitoneal injections for 6 d with 250 μ l of 100 μ M C5 peptide or with PBS. At various time points after peptide injection mice were killed and the thymus was analyzed by FACS®.

FACS® Analysis, Antibodies, and Magnetic Cell Sorting. Analysis was performed on a FACSCAN (Becton Dickinson & Co.) using three-color staining with antibodies conjugated with FITC, PE, or biotin followed by streptavidin-Tricolor (Caltag Laboratories, San Francisco, CA). The transgenic β chain was detected with antibody F23.1 which reacts with V β 8.1, 2, 3 (12). CD4-PE was obtained from Becton Dickinson & Co., and CD8-FITC was prepared by FITC conjugation of antibody YTS169.4 (21). DNA staining was done with 7-aminoactinomycin D (7-AAD¹; Sigma Chemical Co.) as follows: 5 \times 10³ cells in V-bottomed plates were first stained for CD8-FITC and CD4-PE, washed once in PBS containing 0.01% sodium azide, 2% FCS and once again in PBS with 0.3% saponin. 7-AAD (4 μ g/ml in PBS-saponine) was then added and the plates were incubated at room temperature shielded from light for 30 min. Without a further washing step, the samples were then analyzed using linear scale for FL3 acquisition to assess 7-AAD staining.

Positive selection of T cells expressing V α 2 TCR determinants was done by magnetic cell sorting with the Vario-MACS (Miltenyi Biotech, Bergisch Gladbach, Germany) using biotinylated anti-V α 2 (22) antibody and following the procedure recommended in the manual. Positively selected cells were passed over the selection column twice and the purity of the selected population was between 80 and 90%.

Results

Generation of Transgenic Mice with a Class II-restricted TCR Specific for the Serum Protein C5. The A18 T cell clone that donated the receptor used to generate transgenic mice is a high affinity clone that recognizes very small amounts of C5 protein presented in the context of I-E κ MHC (10, 18). The C5 epitope recognized is peptide 107-121 close to the NH₂

¹ Abbreviation used in this paper: 7-AAD, 7-aminoactinomycin D.

terminus of the C5 β chain. The A18 T cell receptor was cloned and cDNAs for the α and β chains were placed under the control of the human CD2 promoter and LCR region. The human CD2 cassette was previously shown to drive T lymphocyte-specific, copy number-dependent, and integration site-independent expression of the class I-restricted TCR F5 (15). Five founders were identified upon injection of A18 α and β constructs into CBA oocytes, varying in the copy numbers of transgene from 1 to 100. One transgenic line, A18.2, with about 40 copies of the α transgene and 30 copies of the β transgene, was selected for the following experiments.

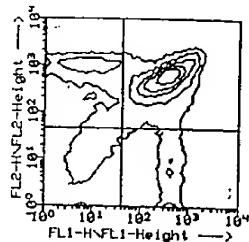
Thymic Development in C5⁻ and C5⁺ Transgenic Mice. The transgenic line A18.2 was selected for further analysis because its TCR expression in the thymus closely followed normal T cell development in nontransgenic mice as illustrated in Fig. 1. In the absence of C5 (C5⁻ Tg⁺) expression

of this class II-restricted receptor results in pronounced skewing of thymocyte development to CD4⁺ cells and virtually no generation of CD8⁺ cells. The TCR, analyzed by staining with the V β 8-specific antibody F23.1 is expressed very low on double negative CD4⁻8⁻ cells, is upregulated to intermediate levels in the double positive CD4⁺8⁺ population, and reaches maximum levels upon maturation to single positive CD4⁺ cells. This receptor development, particularly the fact that double negative thymocytes are TCR^{-/lo}, closely resembles the development in normal mice.

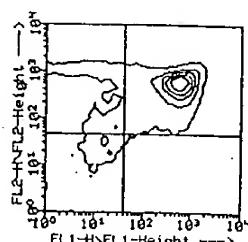
In the presence of C5 (C5⁺ Tg⁺) there is no generation of CD4 single positive cells. In contrast to most class I TCR transgenic mice on a deleting background, however, the number of CD4⁺8⁺ thymocytes is only marginally reduced, which is also reflected in similar total cellularity of the thymus from C5⁺ transgenic mice compared with C5⁻ littermates.

A

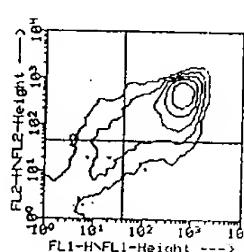
non transgenic



C5-Tg⁺



C5+Tg⁺



B

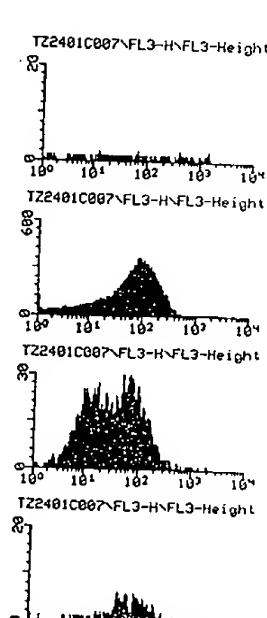
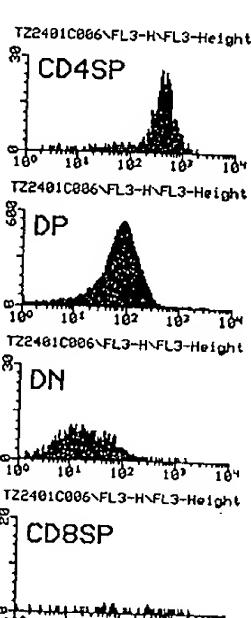
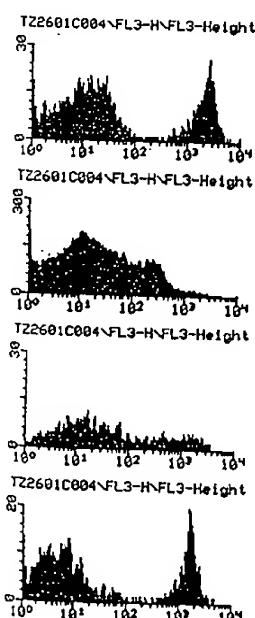


Figure 1. Thymocytes from a nontransgenic mouse, a C5⁻ TCR transgenic mouse, and a C5⁺ TCR transgenic mouse were analyzed for the expression of CD4 and CD8 as well as TCR on gated populations. Staining for TCR was performed with antibody F23.1. (A) Thymic phenotype of transgenic mice in the absence of (C5⁻) or presence (C5⁺) of self-antigen. (B) TCR- β chain expression on gated populations.

A large population of cells with reduced or absent CD4 and 8 expression appears in the thymus of C5⁺ transgenic mice. The apparent TCR expression in the double negative population is probably due to cells that have downregulated TCRs and are in the process of undergoing apoptosis. It appears that tolerance induction to C5 under physiological conditions does not affect the majority of double positive cells, but only those at the transition to single positive cells.

Induction of Apoptosis in Double Positive Cells In Vitro. C5 is a circulating serum protein that has to be internalized and processed by MHC class II expressing APC in the thymus for tolerance induction. Previous studies of the C5 presenting capacity of APC populations in the thymus have indicated that the most efficient APC are thymic dendritic cells (18). This APC population resides in the medulla and the cortico-medullary border which means that the bulk of CD4⁺8⁺ cortical thymocytes may not have access to efficient C5 presentation; this might explain the failure to delete double positive cells. To address this possibility we set up in vitro suspension cultures in which we exposed all thymocytes to dendritic cells as APC in the presence or absence of C5. As previously reported, the contact of thymocytes in vitro with cognate antigen results in apoptosis, which is first visualized by downregulation of CD4 and CD8 on double positive cells (23). As shown in Fig. 2 it was obvious that thymocytes from C5⁻ and C5⁺ transgenic mice initiated apoptosis upon contact with dendritic cells and C5 or C5 peptide whereas non-transgenic control thymocytes remained unaffected. The degree of apoptosis was identical in C5⁻ and C5⁺ thymocytes indicating that both contain the same number of potentially deletable CD4⁺8⁺ thymocytes. When efficient APC can gain access to double positive cells in vitro these cells can be deleted; in vivo we believe this access is restricted. Downregulation of CD4 and 8 correlated with a decrease in DNA staining as measured by triple staining for CD4, CD8, and DNA content (data not shown, but see Fig. 3) indicating that the cells had indeed initiated apoptosis. When transgenic thymocytes from C5⁺ or C5⁻ mice were exposed to macrophages or dendritic cells isolated from thymus of normal C5⁺ animals without any further addition of antigen in vitro it was clear that only dendritic cells could induce apoptosis in agreement with previous findings that indicated that only dendritic cells in the thymus were able to present C5.

Apoptosis of Double Positive Cells In Vivo. The in vitro apoptosis results indicate that double positive cells can be deleted under conditions of optimal antigen presentation. To check whether this is true for double positive cells in vivo, we performed the following experiments. Previous studies with transgenic mice bearing a MHC class II-restricted TCR specific for ovalbumin (7) had shown that peptide injection in vivo resulted in deletion of double positive thymocytes. We performed analogous experiments with the C5 peptide 107-121 recognized by the A18 TCR. Fig. 3 top shows the pattern of CD4 and CD8 expression at different times after daily intraperitoneal injection of C5 peptide (or PBS in the control group). In addition triple staining with the DNA marker 7-AAD, CD4, and CD8 was performed to visualize apoptosis

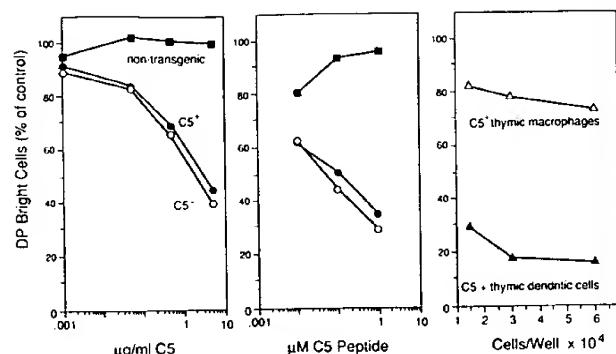


Figure 2. Thymocytes from a C5⁻ (○), a C5⁺ (●) TCR transgenic mouse, and a nontransgenic control (■) were cultured for 12 h with dendritic cells in the absence of C5 or with different doses of C5 protein or C5 peptide. The results shown in the third panel were obtained by culturing thymocytes in the presence of different cell numbers of thymic macrophages or dendritic cells isolated from thymus of normal C5⁺ mice. CD4 and CD8 expression was analyzed by FACS®. The results are expressed as the percentage of bright double positive cells in relation to thymocytes cultured with dendritic cells in the absence of antigen. In the third panel the negative control population consisted of thymocytes without any added cells.

tosis and cell division. Since there are practically no CD8 single positive cells in these mice, all cells that stained positive for CD8 are CD4⁺8⁺ and those that do not stain are CD4 single positive and double negative thymocytes. 7-AAD staining is shown on a linear scale and as indicated in Fig. 3, bottom the top fraction labeled I represents dividing cells in S and M phase with the double content of DNA, fraction II represents cells in G1 phase, and fraction III contains cells with reduced amounts of DNA due to apoptosis. As early as 10 h after peptide injection a significant downregulation of CD4 and 8 molecules can be observed which is paralleled by the appearance of an apoptotic cell population within the double positive cells (characterized by their reduced 7-AAD staining). After 2 d, double positive cells have virtually disappeared and the size of the thymus is reduced from 160×10^6 to 6×10^6 cells. No apoptotic cell population is visible by DNA staining anymore, indicating that deletion and apoptosis have been completed by this time. A seemingly CD8 single positive cell population becomes visible on day 2 and more pronounced on day 6, presumably because of its proportional representation within a thymus devoid of double positive cells. We conclude that in a situation where the requirement for antigen internalization and processing is removed, double positive cells are deleted, indicating that MHC class II positive cells in the cortex are able to present C5 for tolerance induction under these conditions. It should be noted, however, that in the late phase after peptide injection, activated mature CD4⁺ might exacerbate deletion via secretion of mediators such as TNF.

Functional Activity of Mature Peripheral T Cells from C5⁻ and C5⁺ Transgenic Mice. Spleen cells from C5⁻ transgenic mice when cultured with C5 in vitro secrete IL-2 and IFN- γ in response to as little as 10 ng/ml antigen (Fig. 4). We have

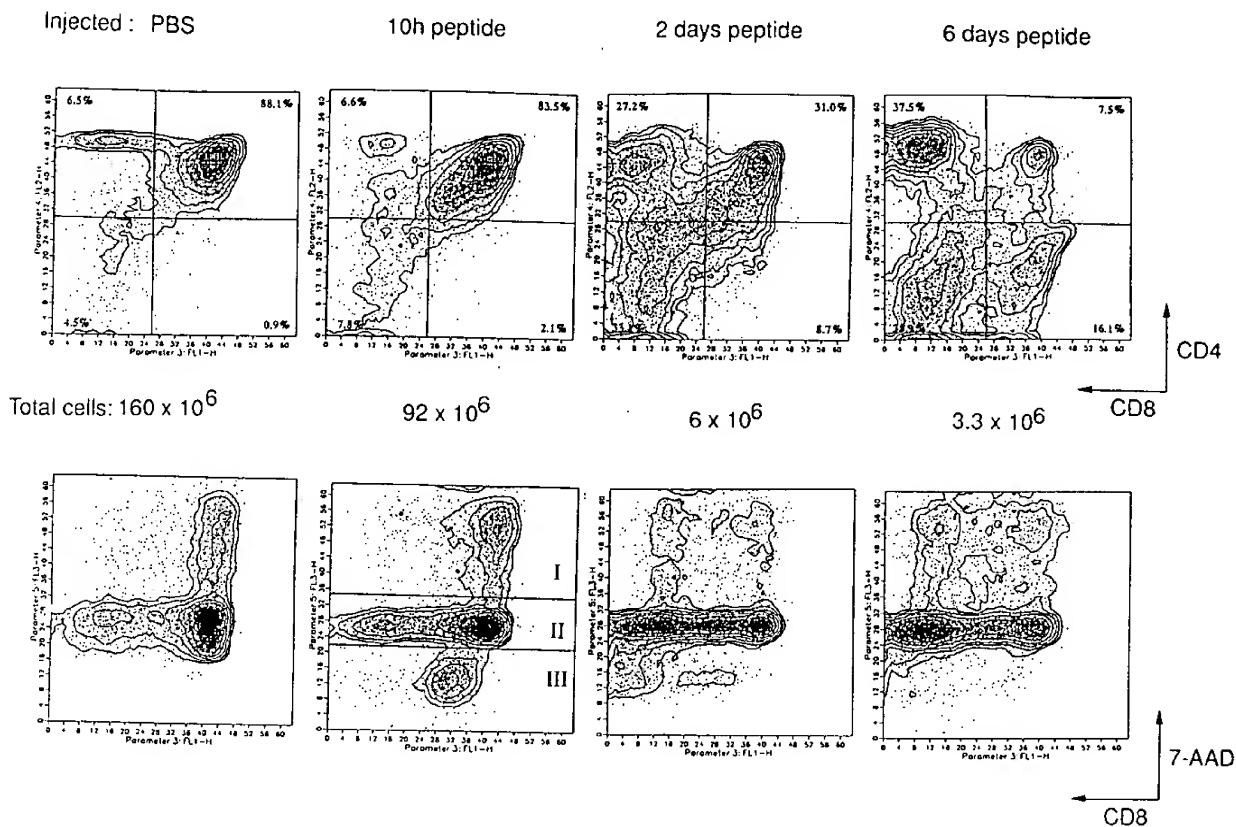


Figure 3. C5⁻ TCR transgenic mice were injected daily with 250 μ l of 100 μ M C5 peptide intraperitoneally for 6 d. At 10 h, 2 d, or 6 d a mouse was killed for analysis of CD4, CD8, and DNA content in thymocytes. The total thymus cell number recovered from each mouse is given. DNA staining by 7-AAD on a linear scale vs. CD8 on a log scale is shown on the bottom. Regions I, II and III refer to cells with double, single, or reduced content of DNA as outlined in Results.

not observed any IL-4 production by these cells. Upon C5 injection, lymph node cells from C5⁻ transgenic mice up-regulate CD69 and CD25 (data not shown). Peripheral T cells from C5⁺ transgenic mice on the other hand do not show signs of activation in vivo. However, they contain cells that can be induced to secrete IL-2 and IFN- γ upon culture with C5 in vitro, indicating that some cells escape tolerance induction in the thymus and are maintained in an anergic state in vivo (Fig. 5). In the absence of a clonotype-specific antibody, we have not been able to identify these cells since endogenous TCR rearrangements that take place in these mice allow accumulation in the periphery of CD4 and CD8 single positive cells that bear nontransgene-derived receptors.

Is C5 Tolerance Induction Incomplete Due to Limited Amounts of Circulating C5 Early in Ontogeny? The question arises how C5-specific T cells escape into the periphery of C5⁺ transgenic mice. An earlier study investigated the postnatal ontogeny of potentially autoreactive cells specific for an Mls^a-encoded determinant. Thymocytes bearing V β 6 were detectable in Mls^a mice neonatally and up to 4 d, but rapidly decreased thereafter (24). We considered the possibility that a delay in optimal C5 expression early in ontogeny might likewise allow some C5-specific CD4 cells to escape from thymic tolerance induction.

C5 synthesis is demonstrable as early as day 10 of gestation (25). However, adult levels of C5 in the circulation are not reached until several weeks after birth. C5 is a serum protein of medium abundance with average levels of 10^{-7} M (about 50 μ g/ml) in adult male mice, and approximately half those levels in female mice (26). Its expression is governed by a single gene and F1 progeny between a C5⁺ and a C5⁻ strain harbor half the concentration of C5 (27). It is also important to note that C5 does not cross the placenta. Given the constraints of age, sex, and haplotype for concentration of the antigen, it is unclear what levels of circulating C5 are found at the time of early T cell differentiation and whether they are sufficient to fully prevent the emergence of mature single CD4⁺ cells. We reasoned that there might be a window in the ontogeny of C5⁺ mice, especially C5^{+/}⁻ mice, in which CD4 single positive cells might be allowed to develop due to limited amounts of self-antigen present for tolerance induction. If that were the case one could assume that the peripheral cells from C5⁺ transgenic mice which were found to be C5 reactive in vitro might have arisen from such an early wave of CD4 cells that had escaped tolerance induction in the thymus.

To test this hypothesis we isolated thymi from mice at day 19 of gestation and tested them for the presence of C5 reac-

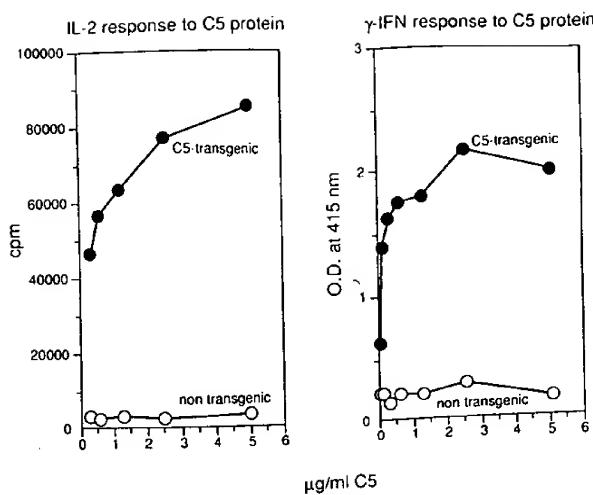


Figure 4. Cells from enzyme digested spleen of a $C5^-$ TCR transgenic mouse and a nontransgenic $C5^-$ control mouse were cultured in 96-well plates with different doses of C5 protein. For analysis of IL-2 production, 75 μ l of culture supernatant were removed after 48 h of culture and tested on the IL-2-dependent CTLL line. Results are expressed as mean cpm (triplicate cultures) of incorporated [3 H]thymidine. For assessment of IFN- γ release 75 μ l supernatant were removed after 72 h of culture and tested in ELISA. The results are expressed as arbitrary units of OD at 414 nm.

tive cells as well as for C5 presentation capacity which was read out as activation of a C5-specific T cell hybrid. Fig. 6 A shows the IL-2 response of thymocytes from individual day 19 fetuses from $C5^-/-$, $C5^{+/-}$, and $C5^{+/+}$ transgenic mice after in vitro culture with dendritic cells and C5. The result unambiguously demonstrates that tolerance induction is complete at day 19 as there is no reactivity in $C5^{+/+}$ or

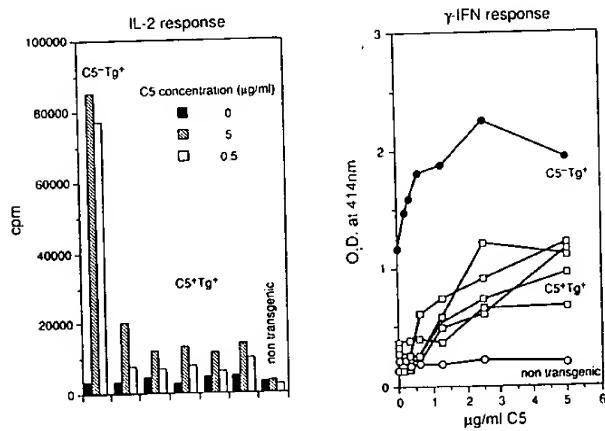


Figure 5. Cells from enzyme digested spleens of a $C5^-$ TCR transgenic mouse, five individual $C5^+$ TCR transgenic mice and a nontransgenic control were cultured in 96-well plates in the presence or absence of different amounts of C5 as indicated. After 48 h of culture, 75 μ l supernatant were removed for analysis of IL-2 activity on the IL-2-dependent CTLL line. Results are expressed as mean cpm (triplicate cultures) of incorporated [3 H]thymidine. For assessment of IFN- γ release 75 μ l supernatant were removed after 72 h of culture and tested in ELISA. The results are expressed as arbitrary units of OD at 414 nm.

$C5^{+/-}$ mice, while C5-specific responses in thymocytes from $C5^-/-$ mice are readily detectable at that time. The reciprocal test for the presence of in vivo processed C5 on day 19 (Fig. 6 B) shows that thymus APC from all $C5^+$ fetuses could be recognized by the C5-specific T cell hybrid A18. There are considerable differences in the levels of stimulation and it is obvious that adult $C5^+$ thymus show higher C5 presentation than day 19 thymus; nevertheless the amounts of C5 present at that time are sufficient to prevent maturation of CD4 single positive cells. This result rules out the possibility that C5-specific T cells in the periphery of $C5^+$ transgenic mice have arisen from an early escape of CD4 single positive cells.

Phenotype of Thymus and Spleen T Cells in $C5^-$ and $C5^+$ Transgenic Mice Crossed onto the $Rag1^{-/-}$ Background. As mentioned previously, endogenous TCR rearrangements that are detectable in $C5^+$ transgenic mice (and to a much lower degree in $C5^-$ littermates) make the identification of potentially autoreactive cells difficult since we do not have a clonotype-specific antibody. In addition we wanted to find out whether suppressor cells are involved in maintenance of C5 tolerance as has been suggested by Cairns et al. (28). We therefore crossed our transgenic mice with mice homozygous for the $Rag1$ gene deletion to prevent rearrangements of endogenous TCR genes so that the only lymphoid cells in this system would be C5-specific T cells. As shown in Fig. 7, $C5^-Rag^-$ transgenic mice show CD4 single positive cells with mature levels of T cell receptor in thymus and the only lymphoid cells found in lymph nodes (and spleen) are CD4 $^+$ T cells with transgenic TCRs. These cells can be activated in vivo and in vitro and behave identically to their CD4 coun-

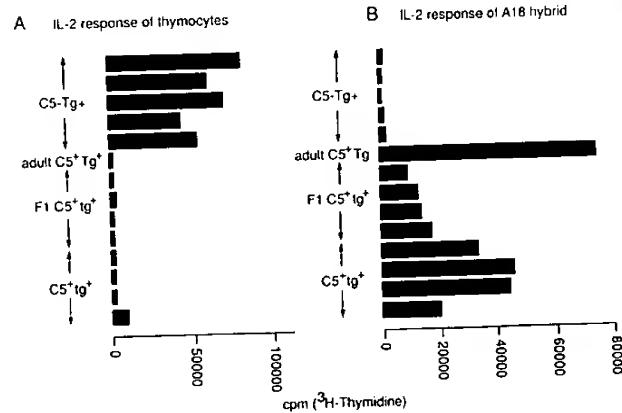


Figure 6. Thymus suspensions from day 19 embryos of $C5^-Tg^+$ (five embryos), $C5^+Tg^+$ (four embryos) or F1 between these two strains (four embryos) and an adult $C5^+Tg^+$ control were prepared by enzyme digestion. In A, 2×10^5 thymocytes/well of a 96-well plate were cultured with 2×10^5 dendritic cells in the presence of 1 μ M C5 peptide for 48 h. 75 μ l of supernatant were then removed and tested for IL-2 activity on CTLL cells. In B, the same thymus cell suspensions were irradiated with 200 Gy and cultured at 2×10^5 cells/well with 5×10^4 cells/well of the A18 T cell hybrid. 24 h later supernatants were transferred to CTLL cells for assessment of IL-2 activity. The results are expressed as mean cpm (triplicate cultures) of incorporated [3 H]thymidine.

terparts in Rag^+ mice indicating that the absence of other lymphoid cells has not prevented the functional development of transgenic T cells. In the thymus of $\text{C5}^+ \text{Rag}^-$ transgenic mice, mature single positive CD4 cells are absent. The number of double positive cells in $\text{C5}^+ \text{Rag}^-$ is slightly decreased compared with C5^- littermates, but the thymic phenotype is very similar to that in young $\text{Rag}^+ \text{C5}^+$ mice. Spleens (and lymph nodes) of $\text{C5}^+ \text{Rag}^-$ mice do not contain any CD4⁺ cells (and in fact no lymphoid cells with expression of Thy1 either) and no functional activity in vitro. These results document that tolerance induction by deletion of MHC class II-restricted T cells specific for circulating C5 is very stringent and that there seems to be no escape of cells through downregulation of TCR or coreceptor.

$\text{C5}^+ \text{Rag}^+$ Transgenic Mice Contain T Cells with Endogenous TCR α Chains and the Transgenic C5 Reactivity. The demonstration of C5-specific T cells that appear in the periphery of $\text{C5}^+ \text{Rag}^+$ transgenic mice, but not $\text{C5}^+ \text{Rag}^-$ transgenic mice implies endogenous TCR rearrangements rescue C5-

specific T cells during selection in the thymus. In this context a crucial question is whether the C5-specific cells in $\text{C5}^+ \text{Rag}^+$ transgenic mice carry a single C5-specific endogenous TCR unrelated to the transgenic receptor or have two receptors, the transgenic one and an additional endogenous receptor that mediated thymic selection. The first possibility is unlikely since a single endogenous receptor conferring C5 specificity would have been subject to tolerization as it is in nontransgenic C5^+ mice. Spleen cells from C5^+ transgenic mice mount a rapid primary C5-specific response in vitro in contrast to normal C5^+ or C5^- mice. It therefore seems reasonable to assume that the transgenic receptor is involved in the response of $\text{C5}^+ \text{Rag}^+$ transgenic mice. The most obvious test for this hypothesis would be double staining for the transgenic TCR $\text{V}\alpha$ chain and any given endogenously derived $\text{V}\alpha$ chain (allelic exclusion for the transgenic TCR β chain is $\geq 90\%$). Unfortunately, the available antibodies against $\text{V}\alpha 11.1$ do not react with the $\text{V}\alpha$ haplotype of the A/J strain (29) so that the transgenic TCR $\text{V}\alpha$ cannot be

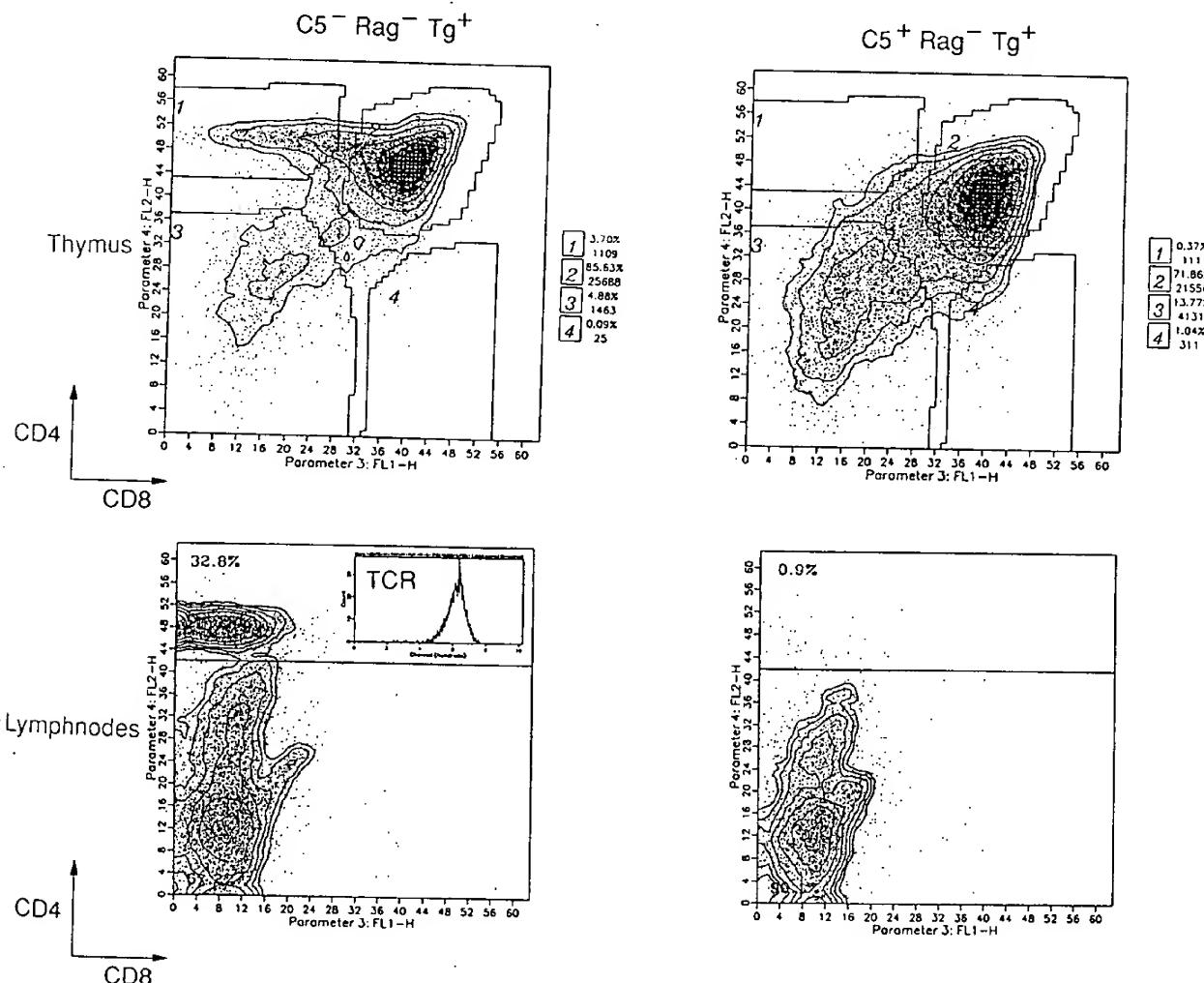


Figure 7. Thymus (top) and lymph node cells (bottom) of a $\text{C5}^- \text{Rag}^-/- \text{Tg}^+$ mouse and a $\text{C5}^+ \text{Rag}^-/- \text{Tg}^+$ mouse were analyzed for CD4 and CD8 expression. The level of TCR expression in CD4⁺ lymph node cells is shown in an inset as assessed by staining with antibody F23.1.

identified by staining. However, the transgenic TCR is easily identified by its reactivity against the A18 peptide epitope of C5. We therefore chose to positively select T cells expressing TCR V α 2 determinants which can be identified with an antibody (22) and test the reactivity of V α 2 expressing T cells to C5 and the A18 peptide in comparison with nontransgenic C5 $^+$ and nontransgenic C5 $^-$ mice.

As shown in Fig. 8, V α 2-expressing T cells, purified from spleen cells of C5 $^+$ transgenic mice by magnetic cell sorting, react to C5 protein and the peptide epitope recognized by the transgenic TCR. No response was detectable in V α 2 T cells from C5 $^+$ nontransgenic mice or in cells from nonimmune C5 $^-$ mice. C5 $^-$ nontransgenic mice immunized with whole C5 protein react to C5 in vitro, but only marginally to the transgenic TCR epitope which is not well represented in the random repertoire of C5 reactive T cells. We therefore conclude that a significant proportion of C5-specific T cells detected in C5 $^+$ Rag $^+$ mice carry V α 2 together with the transgenic receptor (and presumably the transgenic β chain). It is likely that other V α determinants are similarly represented on cells expressing transgenic TCR.

Discussion

This study analyzes the mechanism of tolerance in mice with a transgenic TCR specific for the serum protein C5. C5 is a natural self-protein that causes complete T cell tolerance in mice that express it, whereas no tolerance is induced in C5-deficient strains of mice which lack C5 in their circulation. The basis of tolerance was unknown and suppressor cells were reported to be involved in its maintenance. C5 TCR transgenic mice allowed us to investigate the mechanisms underlying induction and maintenance of tolerance under physiological conditions of antigen expression in vivo by crossing

C5 $^-$ mice that express the transgenic receptor with normal C5 $^+$ mice. The results of these experiments show unequivocally that C5 $^+$ transgenic mice are tolerized by negative selection of CD4 single positive thymocytes. Deletion of self-reactive cells obviously occurs late in thymic ontogeny because the CD4 $^+$ 8 $^+$ population is not depleted in our mice.

In other class II TCR transgenic mice, as far as thymic tolerance induction has been analyzed, deletion of CD4 $^+$ 8 $^+$ thymocytes was observed after injection of cognate peptide. Also transgenic mice bearing a class II-restricted TCR specific for an epitope on the immunoglobulin λ chain showed deletion of double positive cells when these mice were crossed to mice transgenic for the λ chain (30). Since the transgene in the latter mice was expressed under the control of the Ig heavy chain promoter, deletion was attributed to intrathymic synthesis of the λ chain which presumably supplied high local concentrations of antigen rather than to an effect of blood-borne antigen. In fact serum levels of 500 μ g/ml after repeated injections of λ immunoglobulin were required before deletion of double positive cells in the thymus was detectable.

Several possibilities could account for differences observed in intrathymic stages of deletion. First, the level of TCR expression at different stages of T cell ontogeny in the thymus must play an important role in susceptibility to negative selection. The majority of TCR transgenic mice show high levels of TCR expression already at the double negative stage which is not seen in normal mice. This will clearly enhance the chance of negative selection and certainly contributes to the findings that positive and negative selection can occur simultaneously. We chose a transgenic line for this study in which TCR expression follows that seen in normal mice. However, our data disputes the simple notion that insufficient receptor expression caused the delay in negative selection. Clearly double positive cells could be deleted when cultured with dendritic APC in vitro and after injection of the cognate peptide in vivo.

Another possibility is that exposure to negatively selecting antigen is delayed in our mice because it requires internalization and presentation by dendritic cells that are found in the thymic medulla, but not the cortex where the bulk of double positive thymocytes resides (31). Our data do not exclude a role for medullary epithelium (32) as APC for negative selection, but we know that thymic macrophages are incapable of presenting exogenous protein because their levels of class II are very low. Cortical epithelial cells, on the other hand, express high levels of class II molecules, yet seem unable to present C5 for negative selection as judged by the fact that double positive cells are present under physiological conditions in C5 $^+$ mice. This failure to present may be due to their relative inefficiency in internalization of exogenous protein, the absence of costimulatory molecules (33, 34), and/or to the possibility that the cortex does not get access to the full amount of C5 present in the blood circulation. Although proteins have been shown to enter the cortex through the transcapsular route (35), this may not be as efficient as the blood supply which carries circulating proteins into the medulla. Thymic nurse cells as representatives of cortical epithelium have been shown to present intravenous injected pro-

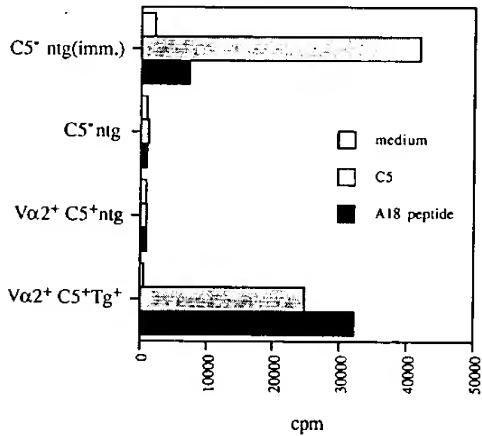


Figure 8. Spleens were taken from C5 $^+$ nontransgenic (ntg) and C5 $^+$ transgenic (Tg $^+$) mice and V α 2-bearing T cells were purified by magnetic cell sorting. Spleens from nontransgenic C5 $^-$ mice were taken from either untreated animals (ntg) or mice primed with C5 in alum 14 d before (imm.). Spleen cells were cultured in the presence of 5 μ g/ml C5 or 1 μ M A18 peptide (107-121) for 48 h. Supernatants were transferred to CTLL cells for assessment of IL-2 activity. The results are expressed as mean cpm (triplicate cultures) of incorporated [3 H]thymidine.

teins, but several milligrams of protein had to be injected to detect its presentation by nurse cells (36). It is quite likely, however, that self-proteins that are more abundant than C5 can get presented by cortical epithelial cells, which might result in the deletion of double positive cells. This interpretation is in agreement with the results in λ light chain-specific TCR transgenic mice. These mice show deletion of double positive thymocytes if the serum concentration of λ chain reaches levels $>500 \mu\text{g/ml}$, which is about 10-fold more than the average C5 concentration.

The conclusion we would like to draw is that the onset of negative selection in the thymus depends on the overall avidity of interactions that are determined partly by TCR expression, and partly by the number of MHC/peptide complexes that are presented. The latter may rarely be limiting for class I-restricted TCRs because of the abundance of class I-expressing cells in the thymus. If the avidity of interactions is high at an early stage of thymocyte development, negative selection will delete the $\text{CD4}^+ \text{8}^+$ compartment. For class II-restricted receptors specific for extracellular antigens, the need for antigen internalization and processing by MHC class II-expressing APC is an obvious limitation to receive high avidity signals for negative selection in the cortical stage of development. When the antigen supply becomes limiting only dendritic cells might be able to provide sufficient MHC/peptide complexes, thus restricting presentation for negative selection to the medulla and cortico-medullary junction and hence a relatively late stage in thymocyte development. Likewise in MHC class I TCR transgenic mice once the antigen density becomes limiting, the elimination of double positive cells is less pronounced (37). Regardless of the delayed onset of negative selection the results presented show that thymic deletion of T cells with C5 specificity is the major mechanism of tolerance induction.

Considering the high incidence of autoimmune disorders with involvement of MHC class II-restricted cells, we had anticipated that negative selection by clonal deletion might be less stringent in this compartment compared with MHC class I-restricted T cells. The inherent limitations of presentation for exogenous antigens, such as threshold concentrations that have to be reached and the need for internalization and processing by MHC class II positive APC raised the possibility that some T cells might escape thymic negative selection. Despite the fact that C5 levels are low early in development and adult amounts of C5 in the circulation are not found until several weeks after birth, there was sufficient self-antigen present to prevent maturation of CD4 single positive cells at day 19. Preliminary experiments using fetal thymic organ cultures have indicated that a day 14 fetal thymus from a C5⁺ transgenic mouse can generate CD4⁺ single positive cells after 10 d of culture indicating that the C5 levels at that time are not high enough for sustained presentation in vitro

in the absence of ongoing C5 supply. Thus, between days 14 and 19 C5 synthesis increases sufficiently to supply the levels of C5 needed for tolerance induction. The demonstration of C5-specific T cells in these mice which are nonresponsive *in vivo*, but are activatable *in vitro* at face value suggested leakiness of the thymic tolerance process. In view of the results with $\text{Rag}^- \text{C5}^+$ transgenic mice in which we do not find any evidence of T cells escaping into the periphery, however, this notion has to be modified. The findings rule out a number of possibilities which could have accounted for the presence of potentially self reactive cells in C5⁺ mice. First, they show that clonal deletion is complete and that there is no significant leakage into the periphery of cells with downregulated receptors or coreceptors, at least up to the relatively young age of 8 wk. Second, the results rule out the contribution of suppressor T cells to the tolerant state since their absence in Rag^- mice should have resulted in accumulation of autoreactive cells. Third, our findings strongly suggest that endogenous rearrangements might be involved in creating a potentially autoreactive repertoire of cells in the periphery.

One possibility is that endogenous rearrangements have created receptors composed of the transgenic β chain with an endogenous α chain that by chance convey C5 specificity. The reason why this possibility seems relatively unlikely is that C5-specific cells detected in C5⁺ transgenic mice have the same fine specificity as the correct transgenic receptor recognizing peptide 107-121, whereas the random repertoire of C5-specific T cells is diverse and does not focus onto this particular epitope. An alternative possibility is that autoreactive cells might carry two receptors, the transgenic receptor and an additional receptor using a different α chain. Incomplete allelic exclusion of the α chain has been demonstrated to result in productive rearrangement of two different α chains in T cell clones, although technical difficulties prevented their detection on the cell surface (38). Recently, the expression of two different TCRs on cells from transgenic mice was described (39). This phenomenon is not restricted to transgenic mice or T cell clones, but appears to occur frequently in normal T cells as indicated by a study that detected two receptors on about 30% of normal human T cells (40). In our study T cells of C5⁺ transgenic mice carrying a TCR- α chain unrelated to the transgenic TCR were shown to react with the fine specificity of the transgenic receptor. This supports the assumption that they express a second receptor that allows their positive selection and exit into the periphery. The signals involved in this process and the mechanisms that (a) keep the transgenic receptor unreactive *in vivo* and (b) allow its activation *in vitro* are presently unknown, but their elucidation should give important insight into the generation of autoimmunity.

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1. **Transplantation:**
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thanks!

Interactions of Viruses with Dendritic Cells: A Double-edged Sword

By Nina Bhardwaj

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Diverse mechanisms are used by viruses to inhibit, block, or evade the immune response (see review in reference 1). These include reduced expression of critical antigenic epitopes (e.g., EBV in latency), genetic variation of class I-restricted CTL epitopes (HIV-1), clonal exhaustion of CTLs (HIV-1, lymphocytic choriomeningitis virus), downregulation of MHC class I and peptide-MHC complex expression (HSV, adenovirus, cytomegalovirus), production of an immunosuppressive cytokine (e.g., IL-10-like factors by EBV), and downregulation of critical cytokines such as IL-12 (measles virus, HIV-1).

Three studies, two published in this issue of *The Journal of Experimental Medicine*, describe a new mechanism whereby virus infection can subvert the immune response (2-4). Measles virus (MV) infection induces dendritic cell (DC) apoptosis and syncytia formation, leading to profound inhibition of IL-12 production by DCs and T cell proliferation. These studies may therefore provide some explanation for the dramatic immunosuppression that is often observed during MV infection. In addition, they highlight the dual and contrasting roles of DCs as potentiators of antiviral immune responses versus facilitators of disease pathogenesis and immunosuppression.

Characteristics of MV Infection

MV is acquired through the respiratory epithelium where it replicates and disseminates throughout the lymphoid system (5). MV binds to a surface receptor, CD46, one of the regulators of complement activation via hemagglutinin (HA), and then fuses with the cell membrane via its fusion (F) protein (6). Syncytia or multinucleated giant cells (Warthin-Finkeldy cells) have been identified in the submucosal regions of the tonsils and pharynx (7), and may be a source of MV that spreads to other organs and tissues throughout the blood stream.

In healthy children, measles infection is generally self-limited, causing primarily a rash and fever. Complications include an otitis media, pneumonia, gastroenteritis, and rare central nervous system syndromes including a postinfectious encephalitis and a delayed subacute sclerosing panencephalitis (5). MV infection can be complicated by a period of immunosuppression that can lead to secondary infections by bacteria and fungi (8). This is especially significant in developing countries where malnutrition compounds the morbidity associated with these opportunistic

infections. Marked and prolonged abnormalities of cell-mediated immunity have been described. They include T cell lymphocytopenia (9), inhibition of delayed-type hypersensitivity responses (10), suppression of recall responses or proliferation to mitogen or alloantigens (11, 12), suppression of antibody production (13), and cell cycle arrest of lymphoid cells in the G1 phase (11, 12). There may also be skewing of the T helper response towards the Th2 phenotype (14). Cell-mediated immunity appears to be critical in controlling measles infection. Both CD4⁺ and CD8⁺ cells have been implicated in the elimination of measles virus, but it is thought that the humoral response is required for reducing viral load (5).

MV Infects DCs. Several sources of DCs have been investigated: mature cells grown from cord blood progenitors in GM-CSF and TNF (3), skin Langerhans cells (3), mature circulating blood DCs (4), and immature DCs derived from blood monocytes exposed to GM-CSF and IL-4 (2). All DC sources were shown to be productively infected with MV, including the Halle and Edmonston strains. 40–100% of DCs infected at multiplicities of infection (MOIs) of 0.05–0.1 expressed the viral proteins HA and F. Infectious particles were produced, albeit at low levels, 2×10^3 PFU/10⁶ DCs. A small proportion of DCs formed syncytia. However, DC integrity and viability became grossly compromised after a 3–4-d culture, secondary to apoptosis, with death approaching levels of 45–70%. All apoptotic APCs expressed nucleoprotein (NP; reference 2). Monocytes produced similar levels of infectious virus to DCs (peaking at day 5 after infection), and also died from apoptosis but did not form syncytia (2).

Enhancement of MV Production in DC-T Cell Cocultures. When MV-infected DCs were cocultured with T cells, a number of striking observations were made. First Fugier-Vivier et al. (2) showed that addition of PMA/ionomycin-activated T cells increased MV production in DCs up to 18-fold. In contrast, infection in monocytes was only increased 4-fold. Second, syncytia formation increased 7–15-fold. The effects were evident shortly (1–2 d) after DC-T cell coculture. Third, although viral replication occurred primarily in DCs (40–50% NP⁺) versus T cells (10% NP⁺), dramatic levels of apoptosis were evident in both APCs and T cells, leading to 90% cell death by 7 d of coculture. It is not known whether the apoptosis is secondary to the expression of TNF-R superfamily and their ligands, including fasL and fas, as described in HIV-1 infection (15).

MV Interferes with DC and T Cell Function. All three studies demonstrated that T cell proliferation was markedly diminished in cocultures of MV-infected DCs and T cells. Fugier-Vivier et al. (2) found that the effect was evident early in the T cell response (1–2 d). The enhanced production of MV and syncytia formation induced in DCs upon contact with T cells was dependent upon CD40, as it could be significantly blocked by anti-CD40L Ab or mimicked with CD40L-transfected fibroblasts (2). Furthermore, IL-12 production by MV-infected DCs was abrogated by 70%. Since DCs are normally induced to synthesize IL-12 through CD40 signaling (16, 17), this suggests that independent pathways triggered by MV infection abrogate this effect. Indeed, it has recently been demonstrated that antibodies to CD46 can inhibit IL-12 production (18). Curiously, UV-irradiated MV partially inhibited cell proliferation (30%) and IL-12 production (20–30%), but did not induce apoptosis of DCs or T cells or syncytia formation (2).

Using a different system, Grosjean et al. (3) demonstrated that the ability of DCs to stimulate naive, CD45RA⁺, CD4⁺ T cells in the allogeneic mixed leukocyte reaction was completely abrogated after MV infection. As few as 30–100 infected cells caused substantial inhibition (>90%) of proliferation by 2×10^4 T cells. Only a 1-h contact was sufficient and, although there was progressive loss of DC viability, T cell viability was not compromised. These findings differ from Fugier-Vivier et al. (2) in that the latter observed extensive death of T cells. One reason for this may be that Grosjean et al. used mature DC populations. Mature DCs are less efficient at permitting HIV-1 replication than immature cells and may also be less permissive for MV (Granelli-Piperno, A., E. Delgado, V. Finkel, W. Paxton, and R. Steinman, manuscript submitted). Furthermore, these investigators used naive CD45RA⁺ cells rather than preactivated cells.

MV Interferes with the Function of Other APCs. Some of the mechanisms postulated to account for MV-induced immunosuppression here have been described before, although with other cells and not DCs as APCs. For example, Schendler et al. (19) showed that proliferation of PBLs in response to a variety of stimuli was significantly impaired after cocultivation with MV-infected, UV-irradiated autologous blood lymphocytes or monocytes. Direct cell-cell contact rather than inhibition by direct virus infection or release of an inhibitory factor was required. Both the MV HA and F proteins appeared to be critical as coexpression of both, but not the individual proteins, in nonlymphoid cells was necessary to suppress T cell proliferation.

Karp et al. demonstrated that MV infection efficiently downregulated IL-12 production in primary human monocytes (18). Relatively few monocytes (<3% of the total number) needed to be productively infected with MV for inhibition of IL-12 production after stimulation with LPS or staphylococcus Cowan strain 1 plus IFN- γ . However, productive infection was not required for the effect, since pulsing with UV-inactivated MV also suppressed IL-12 synthesis. The mechanism of suppression was considered to be directly due to CD46 cross-linking, since incubation of

CD46 with specific monoclonal antibodies or dimerized C3b directly blocked IL-12 production by LPS or staphylococcus Cowan strain 1 plus IFN- γ .

Finally, B cells also succumb to the effects of MV. In an earlier issue, Ravenel et al. (20) reported that recombinant MV NP directly binds to FcRyII on B cells and inhibits polyclonal Ig production by as much as 50%. Thus, MV appears to affect a multitude of cells and cellular functions that lead to suppression of both cell-mediated and humoral immunity.

Role of DCs in MV Infection. How do these observations reconcile the clinical descriptions of immunosuppression after MV infection and the primary role of DCs as stimulators of immune responses? One interesting possibility is that the giant multinucleated cells (Warthin-Finkeldy cells) in the submucosal areas of the tonsils and pharynx are syncytia consisting of DCs and T cells. The DCs that line the mucosal surfaces where there are also many T cells, are the most likely target cell candidates during viral transmission, as suggested for HIV-1 (21). DCs are an important component of the protective immune response to microbes. Strategically located (lungs, skin, gut, liver), DCs are also recruited into the airway epithelium during the inflammatory response to a broad spectrum of stimuli (22). Thus, DCs may be a reservoir for MV infection and a vehicle to transmit the virus to lymphoid cells in draining nodes. It remains to be seen whether DCs can also mediate protective immunity to MV.

DCs as APCs for Antiviral, T Cell-mediated Immunity

In murine systems, DCs were shown to be the most effective APCs for stimulating recall CTL responses to Sendai virus (23), Moloney leukemia virus (23), HSV (24), and influenza virus (25). However, these studies used viruses simply as antigens to illustrate the potency of DCs to induce CD8⁺ CTL responses.

More recent analyses with human cells have monitored the viral life cycle in DCs. One example is influenza virus. Exposure of DCs to influenza virus at MOIs of 2–4 leads to >90% infection, as manifested by expression of the viral proteins HA and nonstructural protein 1 (26). The infection is nontoxic, as viral protein expression is sustained for >2 d with retention of viability. However, little infectious virus is produced. DCs also synthesize substantial amounts of IFN- α after infection, >3 ng/ml per 10^6 cells (Bender, A., M. Albert, A. Reddy, B. Sauter, G. Kaplan, W. Hellman, and N. Bhardwaj, manuscript submitted). Influenza infection of macrophages also results in viral protein expression in a majority of cells (70%), and synthesis of IFN- α . In contrast to DCs, however, macrophages begin to undergo apoptosis within 6–10 h, and most cells die within 24–36 h. During this interval macrophages synthesize low to moderate levels of virus (Bender, A., M. Albert, A. Reddy, B. Sauter, G. Kaplan, W. Hellman, and N. Bhardwaj, manuscript submitted).

Infected DCs, but not macrophages or B cells, can induce substantial recall CTL responses from purified blood CD8⁺

T cells (26). Three pathways for presentation of influenza antigens to CD8⁺ CTLs by DCs have been identified.

Infectious Virus. Relatively few DCs are required to generate CTL responses (stimulator/responder ratios of 1:50–100) and low levels of infection (MOI of 0.02) are sufficient to generate potent CTLs (26). In contrast, infected monocytes are inactive in inducing these CTL responses, but can serve as targets for the CTLs that are induced by DCs.

Nonreplicating Virus. DCs pulsed with poorly replicating heat- or UV-inactivated influenza virus induce equally strong CTL responses to DCs pulsed with live virus (27). When pulsed with inactivated virus, <1% of DCs express viral protein, including nonstructural protein 1 (which is only synthesized in the infectious cycle), indicating that only small amounts of viral antigen are required by DCs to stimulate T cells (27). The binding and fusogenic functions of inactivated influenza virus are intact as assessed by standard hemagglutinating (binding) and hemolytic (fusion) assays. To be optimally effective, the inactivated virus must retain its fusogenic activity to presumably access the cytoplasm of DCs.

Virus-infected Apoptotic Cells. Monocytes and HeLa cells undergo apoptosis after infection with influenza virus, and can be phagocytosed by uninfected DCs. It has now been shown that DCs process viral antigens from the apoptotic cells and acquire the capacity to induce virus-specific CD8⁺ class I-restricted CTL responses (Albert, M., B. Sauter, and N. Bhardwaj, manuscript submitted). This pathway may account for the phenomenon of cross-priming in animal models, whereby antigens from donor cells could be presented by host bystander cells (28).

The role of DCs in stimulating influenza-specific responses may be physiologic since DCs are residents of airway epithelia and can be rapidly recruited here after exposure to pathogens (22).

DCs in Viral Pathogenesis

The HIV-1 system best illustrates the dual role of DCs during virus infection. DCs express the coreceptors required for the entry of HIV-1, that is, CD4 and several chemokine coreceptors like CXCR4 and CCR5 (29). When exposed to low levels of HIV-1, blood-derived DCs transmit a vigorous cytopathic infection to CD4⁺ T cells which is characterized by syncytia formation, virion release, and T cell death by apoptosis (30–32). This is also the case for DCs derived from human skin (33).

There are three striking features of this system. First, DCs exposed to HIV-1 or carrying a relatively low level of proviral DNA, promote extensive viral replication upon interaction with syngeneic T cells in vitro (33, 34). Infec-

tivity of mature DCs alone, either blood or skin, with HIV-1 is low, however, with few full-length reverse transcripts detectable after infection. After a pulse with MOI of 0.05–0.1, <100 copies of full-length transcripts are detected by PCR per 5×10^4 cells. This low level of infection persists for at least 5 d in vitro and is <10–100 fold less than seen with activated T cells (34). Second, infection in this DC–T cell system is independent of antigens or exogenous stimuli such as IL-2. Third, the syncytia that form are heterokaryons of DCs and T cells and are the sources of viral p24 and virion production. Eventually, cell death of the memory T cells ensues (33, 34).

Cells expressing HIV-1 gag proteins have been detected at the surfaces of mucosal lymphoid tissue, specifically the nasopharyngeal tonsil or adenoid. The cells are comprised of multinucleated syncytia expressing the S100 DC marker (21). Memory T cells traffic through extravascular spaces and can encounter tissue DCs in mucosal sites. Exposure to virus here would permit active replication when both cells interact with death of memory T cells. Thus, DCs may directly contribute to viral transmission, disease pathogenesis, and the high level of CD4⁺ T cell death.

DCs could also have a role in eliciting CD8⁺ anti-HIV-1 responses. Given the ability of DCs to present inactivated influenza virus or infected cells undergoing apoptosis (see above), it is possible that they might present defective HIV-1 (the majority of virus in plasma) or apoptotic CD4⁺ T cells to CD8⁺ T cells. So DCs at sites of viral replication may represent a double-edged sword, promoting HIV-1 replication and inducing antiviral resistance.

As Fugier-Vivier et al. point out (2), the effects of MV infection are curiously reminiscent of infection with HIV-1 where (a) only small amounts of virus are necessary to infect DCs (34), (b) low numbers of infected DCs induce extensive HIV-1 replication in cocultures of activated or memory T cells (33), possibly via CD40L (35), (c) apoptosis is induced in infected and bystander cells (15, 31), (d) there is reduced capacity to synthesize IL-12 (36), (e) syncytia form and are sites of extensive viral replication (30, 32, 33), and (f) virus-infected syncytia are prominent in the epithelium of oral lymphoid tissue (21).

The study of viral life cycles in APCs is leading to a new appreciation of the role of DCs in both protective and pathogenic aspects of viral infection. In influenza, new pathways for charging MHC class I molecules on DCs have been ascertained, in HIV-1 infection, routes for virus transmission have been identified, and in measles infection, new but still undefined pathways for immunosuppression have been discovered.

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thanks!

Rapamycin specifically interferes with GM-CSF signaling in human dendritic cells, leading to apoptosis via increased p27^{KIP1} expression

Andrea M. Woltman, Sandra W. van der Kooij, Paul J. Coffer, Rienk Offringa, Mohamed R. Daha, and Cees van Kooten

The longevity of dendritic cells (DCs) is a critical regulatory factor influencing the outcome of immune responses. Recently, we demonstrated that the immunosuppressive drug rapamycin (Rapa) specifically induces apoptosis in DCs but not in other myeloid cell types. The present study unraveled the mechanism used by Rapa to induce apoptosis in human monocyte-derived DCs. Our data demonstrate that granulocyte-macrophage colony-stimulating factor (GM-CSF) preserves DC survival specifically via the phosphatidyl-

inositol-3 lipid kinase/mammalian target of rapamycin (PI3K/mTOR) signaling pathway, which is abrogated by Rapa at the level of mTOR. Disruption of this GM-CSF signaling pathway induced loss of mitochondrial membrane potential, phosphatidyl-serine exposure, and nuclear changes. Apoptosis of these nonproliferating DCs was preceded by an up-regulation of the cell cycle inhibitor p27^{KIP1}. Overexpression of p27^{KIP1} in DCs using adenoviral gene transduction revealed that apoptosis is directly regulated by p27^{KIP1}. Fur-

thermore, both overexpression of p27^{KIP1} and disruption of the GM-CSF/PI3K/mTOR signaling pathway decreased the expression of the antiapoptotic protein mcl-1. This mTOR/p27^{KIP1}/mcl-1 survival seems unique for DCs and may provide novel opportunities to influence immune responses by specific interference with the life span of these cells. (Blood. 2003;101: 1439-1445)

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Introduction

Apoptosis, or programmed cell death, is a physiologic process that is required for the normal development and maintenance of tissue homeostasis.¹ It is an active process that is regulated by gene products, which either block or accelerate programmed cell death. In most cells, the apoptotic program is always ready to be executed unless continuously inhibited by extracellular survival factors.² Apoptosis regulates many aspects of immunologic homeostasis, including initiation, magnitude, and termination of immune responses. Dendritic cells (DCs) play a critical role in the diverse facets of immune regulation, ranging from tolerance induction and the prevention of autoimmunity to the induction of antitumor immunity and the protection against infectious agents.^{3,4}

DCs are the most potent antigen-presenting cells. They play a major role in the uptake, transport, and presentation of antigens and have the unique capacity to stimulate naive T lymphocytes.⁵ In addition to their polarizing capacity on contact with naive T cells,⁴ they can interact with B cells⁶ and natural killer (NK) cells⁷ and thus direct the character of the immune response. Although of possible biologic importance to down-regulate immune responses, apoptosis in DCs has been scarcely investigated. Several different death receptors have been identified on DCs, including Fas (CD95), tumor necrosis factor (TNF) receptor, and TNF receptor-related apoptosis-inducing ligand receptor (TRAIL-R), suggesting a role for death ligand-induced DC apoptosis.⁸⁻¹⁰ DC apoptosis can also be triggered by UVB radiation,¹¹ glucocorticoids, reactive haptens, infectious pathogens, tumor cells, and NK cells.¹² Although recently, specific nuclear factor κ B (NF- κ B) subunits were described to be important for DC survival in mice,¹³ the molecular

mechanisms underlying DC apoptosis have not yet been unraveled. DCs can also actively protect themselves against cell death. Moreover, DCs protect themselves from cytotoxic T lymphocyte (CTL) attack,¹⁴ suggesting that the survival of DCs is an important regulatory mechanism in immune responses.

Previously, we demonstrated that rapamycin (Rapa) specifically induces apoptosis in both monocyte-derived DCs and DCs generated from CD34⁺ precursors but not in monocytes or macrophages.¹⁵ Rapa, which is an immunosuppressive drug introduced to prevent allograft rejection,¹⁶ is extensively studied for its effect on T lymphocytes and is known mainly for its antiproliferative effect.¹⁷ The drug is structurally related to FK506 that also inhibits T-cell proliferation. Although FK506 and Rapa bind to the same intracellular protein, FKBP-12, the resulting complexes interfere with distinct signaling pathways.^{18,19} FK506 inhibits production of interleukin 2 (IL-2), via inhibition of calcineurin, whereas Rapa inhibits growth factor signaling rather than growth factor synthesis.

The Rapa/FKBP12 complex acts to inhibit the activity of mammalian target of rapamycin (mTOR).^{17,20} mTOR is a member of the lipid kinase family with homology to phosphatidylinositol-3 lipid kinase (PI3K). In T cells, Rapa abrogates the IL-2-induced activity of mTOR, thereby blocking G₁-S transition and proliferation. A potential mechanism by which PI3K and mTOR exert their proliferative effects is by down-regulation of the cyclin-dependent kinase (CDK) inhibitor p27^{KIP1}.²¹ However, up-regulation of p27^{KIP1} is not only linked to cell cycle arrest in G_{0/1},²² but it seems also associated with apoptosis induced by growth factor withdrawal or PI3K inhibition.^{23,24}

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In the present study we examined the mechanism used by Rapa to induce apoptosis in primary human DCs. Our data demonstrate that the survival of monocyte-derived DCs, which are nonproliferating cells, requires granulocyte-macrophage colony-stimulating factor (GM-CSF) signaling via the PI3K/mTOR signaling pathway. Inhibition of mTOR via Rapa leads to increased expression of the cell cycle inhibitor p27^{KIP1} in nonproliferating DCs. Overexpression of p27^{KIP1} by using adenoviral gene transduction was shown to be directly responsible for the down-regulation of the antiapoptotic bcl-2 family protein mcl-1 and the induction of apoptosis.

Materials and methods

Reagents

LY294002 (Alexis, San Diego, CA) and PD98059, SB203580, and Rapamycin (all from Calbiochem, Cambridge, MA) were dissolved in dimethyl sulfoxide (DMSO) and used at the concentrations indicated. FK506 (Prograft; Fujisawa Benelux, Houten, The Netherlands) was added to the cultures at 5×10^{-6} M.

Cell culture

Monocyte-derived DCs were generated as described previously.²⁵ In brief, human monocytes were isolated from a buffy coat obtained from healthy donors using Ficoll-Hypaque (Sigma, St Louis, MO) and Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation, followed by plastic adherence (2 hours) in T75 culture flasks (15×10^6 cells/flask; Nunc/Life Technologies, Breda, The Netherlands). Adherent monocytes were cultured in RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS) and penicillin/streptomycin (P/S) supplemented with 5 ng/mL GM-CSF (Sandoz, Uden, The Netherlands) and 10 ng/mL IL-4 (Sanvertech, Heerhugowaard, The Netherlands) for 6 days. Differentiation into CD1a⁺CD14⁻CD83⁺ immature DCs was confirmed by fluorescence-activated cell sorter (FACScan) analysis.

Induction and detection of apoptosis

Apoptosis induction experiments were performed in 12- or 24-well culture plates (Costar, Cambridge, MA) containing 5×10^5 DC/mL.

Determination of nuclear condensation and fragmentation. Cells were harvested at the indicated time points and fixed with 1% paraformaldehyde on ice. Cytopsin preparations were made and stained with Hoechst 33258 (Molecular Probes, Leiden, The Netherlands) for 3 minutes at room temperature. The percentage of apoptotic cells was determined by examining 200 cells and counting the cells that were characterized by condensed or fragmented nuclei.

Determination of phosphatidyl-serine exposure. Cells were harvested at the indicated time points, washed, labeled with annexin V-fluorescein isothiocyanate (FITC; Apoptest FITC kit; Nexins Research BV, Kattenijke, The Netherlands) for 30 minutes on ice, and subsequently taken up in 1 μ g/mL propidium iodide (PI; Molecular Probes). Annexin V/PI staining was conducted on a FACScan and analyzed using WinMDI software (Becton Dickinson).

Determination of DNA fragmentation (sub-G₀ analysis). Cells were harvested, washed in 1 mM EDTA/PBS (ethylenediaminetetraacetic acid/phosphate-buffered saline), and fixed in 90% ethanol for at least 30 minutes at -20°C . Then the cells were washed, and cellular DNA was stained by treating the cells with 50 μ g/mL RNase A (Sigma) and 10 μ g/mL PI (Molecular Probes) for 45 minutes at room temperature. Fluorescence intensity was quantified on a per cell basis by flow cytometry (FACScan).

Determination of mitochondrial transmembrane potential. Mitochondrial dysfunction was assessed by using rhodamine 123 (Rh123; Molecular Probes). Cells were incubated at 37°C for 30 minutes in the presence of 0.1 μ g/mL Rh123. Then cells were washed and resuspended in PBS, either with or without 1 μ g/mL PI, and Rh123 retention was conducted on a FACScan.

Preparation of whole cell lysates and Western blot analysis

Cultured DCs were harvested, washed, and lysed in Triton X-100 buffer, containing 20 mM Tris (tris(hydroxymethyl)aminomethane)-HCl (pH 7.4), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 2 μ g/mL leupeptin, 2 μ g/mL antipain, 2 μ g/mL chymostatin, and 5 U/mL trypsin. The protein concentration was determined by using the bicinchoninic acid (BCA) protein assay (Pierce Chemical, Rockford, IL). Each protein sample was separated under reducing conditions on a 12% polyacrylamide sodium dodecyl sulfate (SDS) gel and semi-dry electroblotted onto polyvinylidene fluoride (PVDF) membranes (Immobilon-p; Millipore, Bedford, MA). Membranes were incubated with 2% casein in PBS-0.05% Tween 20 for blocking, and then the primary antibody, either mouse-antihuman p27^{KIP1} (F-8; Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit-antihuman mcl-1 (S-19; Santa Cruz Biotechnology), was added. After incubation with the appropriate secondary antibody (horseradish peroxidase [HRP]-conjugated swine-antirabbit immunoglobulin or goat-antimouse immunoglobulin both from Dako, Glostrup, Denmark) detection was performed with Supersignal (Pierce), and the blots were exposed to Hyperfilm films (Amersham Pharmacia Biotech, United Kingdom). Membranes were stripped by using Restore Stripping Buffer (Pierce) to investigate the expression of several specific proteins within one experiment. Equal protein loading was verified by Coomassie blue staining of the blot, which allows comparison over the whole molecular weight range.

Recombinant adenoviral vectors

Replication-deficient adenoviral vectors containing either human p27^{KIP1} cDNA (rAd-p27) or the LacZ gene (rAd-LacZ)²⁴ were obtained from Drs I. Naruse and H. Hoshino (Gunma University School of Medicine, Maebashi, Japan). Adenoviral stocks were generated and purified by double CsCl density gradient centrifugation as described previously.²⁶ To remove the CsCl, the virus bands were dialyzed against a Tris buffer (25 mM Tris-Cl, 137 mM NaCl, 5 mM KCl, 0.73 mM Na₂HPO₄, 0.9 mM CaCl, 0.5 mM MgCl₂, pH 7.45). The final dialysis was performed with this Tris buffer containing 5% sucrose, and then virus stocks were stored at -80°C until further use.

Infection of DCs with rAd

Day 6 immature DCs (0.4×10^6) were resuspended in 100 μ L PBS and incubated with rAd-LacZ (2.0×10^{10} pfu/mL) or rAd-p27 (1.2×10^{10} pfu/mL) (multiplicity of infection [MOI], 1000).²⁷ After 2 hours at 37°C , DCs were washed twice with PBS to remove free adenoviruses. Then cells were resuspended in RPMI 1640 containing 10% AFCS and P/S supplemented with 5 ng/mL GM-CSF and 10 ng/mL IL-4 and cultured at 37°C in a 5% CO₂ incubator.

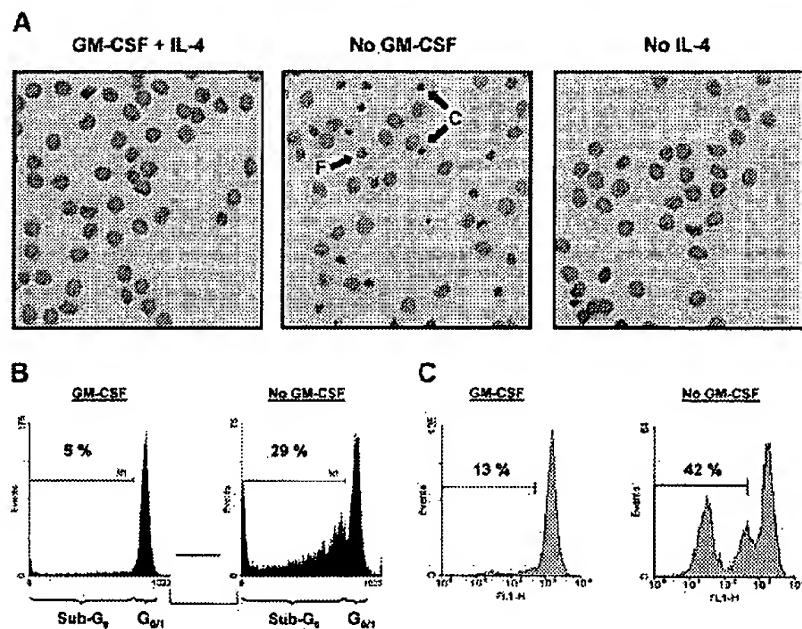
After 24 hours of culture, DCs were fixed with 0.2% glutaraldehyde/2% formaldehyde in PBS for 5 minutes at 4°C . Fixed cells were incubated in a stain solution containing 5 mM potassium ferrocyanide²⁺, 5 mM potassium ferrocyanide³⁺, 2 mM MgCl₂, and 1 mg/mL X-gal (Sigma) for 4 hours at 37°C . The percentage of LacZ⁺ cells (blue cells) was calculated by light microscopy.

Results

Monocytes and DCs differ in their survival mechanisms

As demonstrated previously, addition of GM-CSF to monocyte cultures increased the yield of functional immature DCs on culture with IL-4.²⁸ To examine whether GM-CSF increases cell recovery by affecting cell survival at the start of culture, ie, an effect on freshly isolated monocytes, or by promoting DC survival, both monocytes and DCs were cultured with or without GM-CSF for 48 hours. GM-CSF withdrawal, but not IL-4 withdrawal, from DC cultures strongly induced apoptosis (Figure 1). GM-CSF withdrawal induced the typical characteristics of apoptosis, including

Figure 1. GM-CSF withdrawal induces apoptosis in DCs. DCs were cultured as described in "Materials and methods." At day 6, DCs were harvested, extensively washed, and further cultured in RPMI-10% FCS supplemented with IL-4 (10 ng/mL) plus GM-CSF (5 ng/mL; designated as "GM-CSF" in panels B and C), IL-4 alone (10 ng/mL; designated as "No GM-CSF"), or GM-CSF alone (5 ng/mL; designated as "No IL-4"). After 48 hours of incubation, cells were analyzed for the amount of apoptosis using Hoechst to analyze nuclear morphology (A), PI to detect DNA fragmentation (B), or Rh123 to investigate $\Delta\Psi_m$ (C). Photographs (original magnification, $\times 400$) taken from cells stained with Hoechst were inverted and demonstrate condensed ("C") and fragmented nuclei ("F") indicated by arrows (A). Data presented are representative of 5 independent experiments with different donors.



nuclear condensation and fragmentation visualized by Hoechst staining (Figure 1A) and DNA fragmentation as demonstrated by an increased sub-G₀ fraction after DNA staining with propidium iodide (Figure 1B). The loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) on GM-CSF deprivation, as determined by Rh123 retention, suggested an active role of mitochondria in the induction of apoptosis (Figure 1C).

Both mitogen-activated protein kinase (MAPK) and PI3K/mTOR signaling pathways have been suggested to play a role in GM-CSF-induced survival of various hematopoietic cells. To investigate the involvement of the distinct signaling pathways in GM-CSF-mediated survival of DCs, the activation of the MAPK family members extracellular signal-related kinase (ERK) and p38, PI3K, and mTOR was blocked by culturing DCs in the presence of PD98059, SB203580, LY294002, and Rapa, respectively. PD98059 slightly induced apoptosis, whereas inhibition of p38 by SB203580 did not (Figure 2A). Both inhibition of PI3K using LY294002 and inhibition of mTOR using Rapa induced apoptosis comparable to GM-CSF deprivation, demonstrating a critical role for PI3K/mTOR signaling in the regulation of DC survival. Inhibition of PI3K, ERK, or p38 in monocytes slightly induced apoptosis (Figure 2A). As shown previously,¹⁵ monocytes were completely insensitive for the proapoptotic activity of Rapa, demonstrating that the longevity of DCs and their precursors, ie, the monocytes, are regulated by different survival mechanisms.

DC survival requires GM-CSF signaling via PI3K and mTOR

Like treatment of DCs with Rapa to inhibit mTOR,¹⁵ blockade of PI3K in DCs by addition of LY294002 time- and dose-dependently induced apoptosis that was characterized by nuclear condensation and fragmentation (Figure 2B) and DNA fragmentation (data not shown). In addition to the data obtained from GM-CSF deprivation experiments, both LY294002 and Rapa demonstrated loss of $\Delta\Psi_m$ associated with the percentage of apoptosis induced (Figure 2C). In line with the induction of apoptosis, inhibition of ERK by PD98059 only slightly induced loss of $\Delta\Psi_m$, and inhibition of p38 did not significantly change $\Delta\Psi_m$ (Figure 2C).

Rapa increases the expression of $p27^{kip1}$

Because mTOR is the most downstream target in GM-CSF-driven survival of DCs as determined so far, and because Rapa is clinically used as an immunosuppressive drug, we further explored the mechanism used by Rapa to specifically induce apoptosis in DCs. In T lymphocytes, Rapa exerts its effects via the up-regulation of the cell cycle inhibitor $p27^{kip1}$. An increased expression of $p27^{kip1}$ in nonproliferating eosinophils has been shown to correlate with the induction of apoptosis.²³ Therefore, we investigated whether Rapa or GM-CSF signaling could influence $p27^{kip1}$ protein expression in monocyte-derived DCs, which are also nonproliferating cells. A 48-hour incubation in the presence of Rapa strongly increased the expression of $p27^{kip1}$ (Figure 3A). When DCs were simultaneously treated with FK506, which antagonizes the apoptotic effect of Rapa (Figure 3B), the Rapa-induced $p27^{kip1}$ expression also could be inhibited (Figure 3A). Although Rapa required more than 24 hours to induce strong apoptosis,¹⁵ the effect on the expression of $p27^{kip1}$ was observed relatively early. Treatment with Rapa for 5 hours already increased the expression of $p27^{kip1}$ that was not further increased by an extended incubation period in the presence of the drug (Figure 3C-D).

$p27^{kip1}$ expression is increased after GM-CSF withdrawal or PI3K Inhibition

To support the hypotheses that Rapa-induced apoptosis is mediated by an increased $p27^{kip1}$ expression, we analyzed the effect of cytokine withdrawal and PI3K inhibition on the expression of $p27^{kip1}$. All culture conditions that lead to apoptosis of DCs, including cultures without GM-CSF or with the PI3K inhibitor LY294002, were accompanied by an increased $p27^{kip1}$ expression, whereas no changes were observed in conditions without apoptosis, such as IL-4 withdrawal or DMSO (Figure 4A).

Monocytes were completely resistant to Rapa-induced apoptosis (Figure 2A). Rapa did not increase $p27^{kip1}$ in cultured peripheral blood monocytes (Figure 4B), confirming the hypothesis that

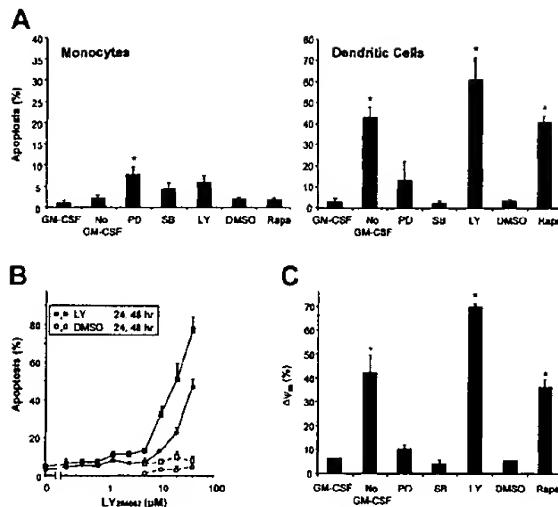


Figure 2. Monocytes and DCs differ in their survival mechanisms. (A) DCs were cultured in RPMI-10% FCS with the addition of IL-4 and GM-CSF, whereas freshly isolated peripheral blood monocytes were cultured in RPMI-10% FCS alone. After 48 hours of culture, the effects of GM-CSF deprivation (No GM-CSF) or treatment with PD98059 (50 μ M), SB203580 (10 μ M), LY294002 (20 μ M), Rapa (1 μ M), or DMSO, were analyzed using Hoechst. Results demonstrate mean \pm SD percentage of apoptosis of 3 independent experiments with different donors. * indicates significant apoptotic effect of "No GM-CSF" compared with "GM-CSF" or an inhibitor compared with its solvent "DMSO" ($P < .05$; Student *t* test for paired samples). (B) DCs were cultured with IL-4 and GM-CSF in the presence of various concentrations of the PI3K-inhibitor LY294002, or DMSO. Cells were analyzed after 24 hours or 48 hours using Hoechst ($n = 2$). (C) Day-6 DCs were harvested, extensively washed, and further cultured in RPMI-10% FCS supplemented with IL-4 plus GM-CSF (control), IL-4 alone (No GM-CSF), or IL-4 plus GM-CSF with either Rapa (1 μ M), LY294002 (20 μ M), PD98059 (50 μ M), or SB203580 (10 μ M) added. After 48 hours, $\Delta\Psi_m$ was investigated using Rh123. The percentage of cells with a decreased $\Delta\Psi_m$ are plotted in a histogram. Data are shown as the mean \pm SD percentage of apoptosis of duplicate cultures, representative for 3 to 6 independent experiments with different donors. * indicates significant apoptotic effect compared with "GM-CSF" or "DMSO" ($P < .05$; Student *t* test for paired samples).

Rapa-induced apoptosis is preceded and accompanied by an increased expression of p27^{KIP1}.

p27^{KIP1} gene transduction induces apoptosis in DCs

To determine whether up-regulation of p27^{KIP1} has a causative role in Rapa-induced apoptosis of monocyte-derived DCs, DCs were

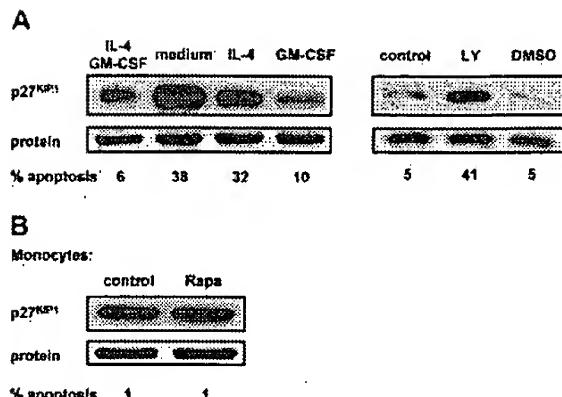


Figure 4. p27^{KIP1} protein levels correlate with the induction of apoptosis. (A) Day-6 DCs were harvested, extensively washed, and further cultured in RPMI-10% FCS in the presence or absence of IL-4 and GM-CSF (left panel) or in the presence of both IL-4 and GM-CSF with or without LY294002 (20 μ M) or DMSO (right panel). Data demonstrated in left and right panels are derived from different experiments with different donors. (B) Freshly isolated peripheral blood monocytes were cultured in RPMI-10% FCS with or without addition of Rapa (1 μ M).

infected with recombinant adenoviruses (rAd) containing human p27^{KIP1} cDNA. Infection of DCs with rAd-LacZ served as a control and showed that transduction efficiencies of above 90% were achieved at an MOI of 1000 (Figure 5A). Expression of p27^{KIP1} protein after infection with the adenoviral vectors was measured by Western blot analysis. A strong overexpression of p27^{KIP1} was detected at 48 hours after rAd-p27 infection (Figure 5B), which was not further increased after 72 hours. Expression of p27^{KIP1} protein in DCs infected with rAd-LacZ was not different from control levels.

Cells were harvested after 48 hours of incubation. Whole cell lysates were prepared, equal amounts of protein (20 μ g/lane) were loaded, and the levels of p27^{KIP1} were determined. Data shown are representative for 3 independent experiments with different donors.

In the presence of GM-CSF, DCs infected with rAd-p27 went into apoptosis as demonstrated by an increased annexin V binding within the PI⁻ population (Figure 5C) and nuclear condensation and fragmentation (data not shown). Kinetic experiments showed that p27^{KIP1}-induced apoptosis of DCs was already present 24 hours after infection, which further increased

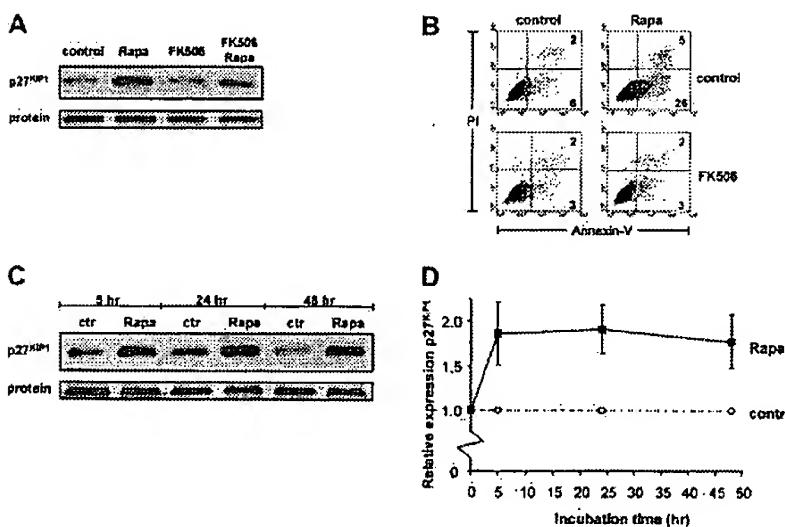
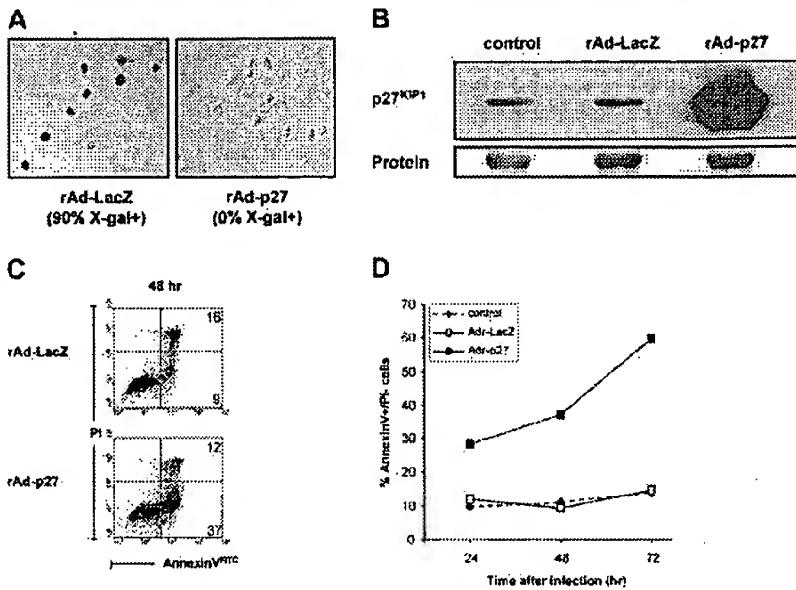


Figure 3. Rapa increases expression of p27^{KIP1} protein. (A-B) DCs were cultured with IL-4 and GM-CSF in the absence or presence of Rapa (10⁻⁷ M) and either with or without addition of FK506 (5 \times 10⁻⁶ M) for 48 hours. Part of the cells was used to prepare whole cell lysates. Equal amounts of protein (20 μ g/lane) were loaded, and the levels of p27^{KIP1} were determined (A). Apoptosis was detected in remaining cells by flow cytometry, using annexin V-FITC and PI staining (B). Data shown are representative for 3 independent experiments performed with different donors. (C-D) DCs were cultured in IL-4 and GM-CSF with or without addition of 1 μ M Rapa. Cells were harvested after 5 hours, 24 hours, or 48 hours of incubation. Whole cell lysates (20 μ g/lane) were loaded, and the levels of p27^{KIP1} were determined by Western blot analysis (C). The relative expression of p27^{KIP1} was determined by using Stratagene-EagleSight (La Jolla, CA) and represents the ratio of each band from Rapa-treated DCs to that of untreated DCs. Results are expressed as mean ratio (\pm SD) of 4 independent experiments (D).

Figure 5. Overexpression of p27^{KIP1} induces apoptosis in DCs. (A) Evaluation transduction efficiency of rAd-LacZ or rAd-p27-infected immature DCs using X-gal. (B) DCs were infected with rAd-LacZ or rAd-p27. After 48 hours of culture in the presence of IL-4 and GM-CSF, infected DCs and control DCs were harvested for the preparation of whole cell lysates. Then equal amounts of protein (20 µg/lane) were loaded, and the levels of p27^{KIP1} were determined. Data shown are representative for 3 independent experiments with different donors. (C) DCs were infected with rAd-LacZ or rAd-p27 and subsequently cultured in IL-4 and GM-CSF. After 48 hours of culture, annexin V-FITC/PI staining was performed to determine the percentage of apoptotic cells. Data shown are representative for 2 independent experiments with different donors. (D) DCs were left untreated or infected with rAd-LacZ or rAd-p27 and subsequently cultured in IL-4 and GM-CSF. Cells were harvested after 24, 48, and 72 hours of culture. Annexin V-FITC/PI stainings were performed to determine the percentage of annexin V⁺/PI⁻ (early apoptotic) cells ($n = 2$).



in time (Figure 5D). No differences were found in annexin V binding and nuclear morphology between rAd-LacZ-infected DCs and control DC cultures.

Increased expression of p27^{KIP1} is responsible for down-regulation of mcl-1

As demonstrated earlier, apoptosis of DCs induced as a consequence of a disruptive GM-CSF/PI3K/mTOR signaling pathway is

associated with loss of mitochondrial integrity. The bcl-2 family of proteins, containing both antiapoptotic and proapoptotic members, controls mitochondrial permeability and thus plays a critical role in the regulation of apoptosis. mcl-1 is an antiapoptotic protein, which is thought to be an important bcl-2 family member in GM-CSF-mediated survival of hematopoietic cells.²⁹

Western blot analysis of lysates prepared after rAd-p27 infection demonstrated that increased expression of p27^{KIP1} directly causes down-regulation of mcl-1 protein expression in DCs (Figure 6A). The correlation between the down-regulation of mcl-1 and the interference in the GM-CSF signaling pathway at the level of PI3K and mTOR finally leading to apoptosis was further examined. Addition of either LY294002 or Rapa to DC cultures, as well as deprivation of GM-CSF, induced a down-regulation of mcl-1 that correlated with the percentage of apoptosis induced, thereby demonstrating that expression of mcl-1 is tightly regulated via PI3K and mTOR in response to GM-CSF (Figure 6B).

A more detailed analysis of the effect of Rapa on the expression of mcl-1 in DCs was performed. The Rapa-induced reduction of mcl-1 protein levels was already observed within 24 hours of incubation, as demonstrated by Western blot analysis, but became more pronounced after 48 hours of treatment, finally resulting in a 2-fold reduction in mcl-1 protein levels (Figure 6C).

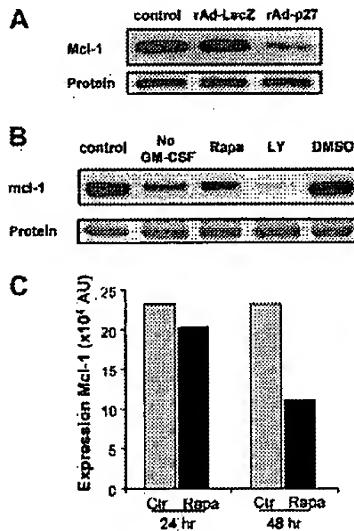


Figure 6. Overexpression of p27^{KIP1} as well as disruptive GM-CSF/PI3K/mTOR signaling down-regulate mcl-1 protein expression. (A) DCs were left untreated or infected with rAd-LacZ or rAd-p27. After 48 hours of culture in the presence of IL-4 and GM-CSF, DCs were harvested for the preparation of whole cell lysates. Then equal amounts of protein (25 µg/lane) were loaded, and the levels of mcl-1 were determined. Data shown are representative for 2 independent experiments with different donors. (B) DCs were cultured either in IL-4 (No GM-CSF) alone or IL-4 and GM-CSF. Cultures containing IL-4 and GM-CSF were supplemented with or without 10 µM LY294002, 1 µM Rapa, or DMSO. Equal amounts of protein (20 µg whole cell lysate/lane) were loaded, and the levels of mcl-1 were determined. Data shown are representative for 3 independent experiments performed with different donors. (C) Quantification of mcl-1 expression in control and Rapa-treated DCs after either 24 hours or 48 hours of culture as described in "Materials and methods." Data shown are representative for 4 independent experiments performed with different donors.

Discussion

In the present study we show that defective GM-CSF signaling in human nonproliferating DCs, either because of selective inhibition of PI3K or mTOR, or GM-CSF deprivation, caused an increased expression of the cyclin-dependent kinase (CDK) inhibitor p27^{KIP1} prior to a decreased expression of the antiapoptotic protein mcl-1 and apoptosis. Overexpression of p27^{KIP1} in DCs by using adenoviral gene transduction revealed that both mcl-1 expression and cell survival are directly regulated by p27^{KIP1}.

By examining the mechanism used by Rapa for the induction of apoptosis in DCs, we found that the PI3K/mTOR pathway is a critical signaling route for GM-CSF-driven survival of monocyte-derived DCs. GM-CSF is a potent growth factor for a variety of hematopoietic cells. On binding to its heterodimeric receptor,

GM-CSF activates several signaling pathways, including the Jak-Stat (Janus kinase and signal transducer and activator of transcription) and Ras pathways,^{30,31} resulting in both mitogenic and antiapoptotic signals. Although downstream from Ras, both Raf/MAPK and PI3K/mTOR pathways have been suggested to play a role in cytokine-driven survival of hematopoietic cells,^{31,32} we found that for monocyte-derived DCs only the PI3K/mTOR pathway is absolutely necessary. In line with a previous study on lipopolysaccharide (LPS)-stimulated monocyte-derived DCs,³³ which also demonstrate a cell death-inducing effect of LY294002 but not of PD98059 or SB203580, we observed only a limited role for ERK activation in DC survival. Although our data suggest that Rapa specifically counteracts the antiapoptotic activity of GM-CSF that results in an increased expression of p27^{KIP1}, the fact that Rapa further increases apoptosis on GM-CSF withdrawal (data not shown) might suggest additional modes of action of Rapa.

p27^{KIP1} is an inhibitor of cell cycle progression, exerting its effect through interaction with cyclin-CDK complexes and arresting cells in G_{0/1}.²² p27^{KIP1} is present at relatively high levels in quiescent cells and is down-regulated by mitogenic stimulation^{21,34,35}, the latter process being blocked by Rapa.²¹ It is important to note that a cell cycle inhibitor can also play a decisive role in a nonproliferating cell type such as DCs or, as shown, nonproliferating eosinophils.²³ However, although both in eosinophils and in the murine IL-3-dependent cell line Ba/F3 apoptosis was linked to increased p27^{KIP1} and interference with the PI3K pathway, apoptosis could not be induced by Rapa.²³ This finding further underlines the specificity of Rapa toward DC apoptosis. It has been shown that p27^{KIP1}-deficient mice demonstrate an increased survival of bone marrow-derived stem cells when cultured ex vivo compared with wild-type mice,²³ but the immune status has not been specifically investigated.³⁶ The mechanism by which p27^{KIP1} can regulate cell survival is not known. In search of a potential mechanism by which p27^{KIP1} could modulate GM-CSF-driven DC survival, we focused on the regulation of mcl-1.

mcl-1, initially cloned as a gene differentially expressed in human ML-1 myeloid leukemia cells,³⁷ shows extensive homology to bcl-2. Like bcl-2 and bcl-x_L, mcl-1 heterodimerizes with bax³⁸ and thus plays an important role in the prevention of apoptosis. Although mcl-1 and bcl-2 show strong homology, their distribution, expression levels, and regulation of apoptosis are independently regulated.^{39,41} mcl-1 is thought to be an important protein in IL-3 and GM-CSF-mediated survival of hematopoietic cells,^{29,42} a

finding supported by the fact that mcl-1 transgenic mice possess an enhanced viability in a wide range of hematopoietic cell types.⁴³

We demonstrate that the protein level of mcl-1 in DCs is tightly regulated by GM-CSF signaling via PI3K and mTOR. Despite the large decrease in the level of mcl-1 protein following treatment with Rapa or LY294002, no significant decrease in the level of mcl-1 mRNA was observed (data not shown). This finding is consistent with previous work demonstrating that GM-CSF-mediated mcl-1 expression is regulated at the translational level by a PI3K-controlled pathway.⁴⁴ LY294002-treated human monocyte-derived macrophages⁴⁵ showed also a marked decrease of mcl-1 protein expression, but they appear to have different PI3K/mcl-1 survival mechanisms than monocyte-derived DCs, because monocytes and macrophages do not undergo Rapa-induced apoptosis (Figure 2).¹⁵ In addition, we showed that monocytes do not increase their p27^{KIP1} expression on Rapa treatment, whereas DCs clearly do. Therefore, alternative survival mechanisms might be present in monocytes/macrophages that regulate the expression of mcl-1 independently of mTOR and p27^{KIP1}.

In conclusion, given their central role in the immune system, DCs are important targets for both immunosuppressive or immunostimulatory therapies.⁴⁶⁻⁵⁰ Understanding the survival program of DCs will provide the opportunity to either increase immune responses by prolonged DC survival or to terminate these responses by specific depletion of the cells. Rapa, which has been introduced recently as an effective drug to prevent allograft rejection, might partially exert its immunosuppressive effect by virtue of its proapoptotic effect on DCs. Our finding that the survival of monocyte-derived DCs is specifically regulated by the GM-CSF signaling pathway via PI3K/mTOR and involves the regulation of p27^{KIP1} and mcl-1 might provide additional tools to control immune responses.

Acknowledgments

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1. Transplantation:
2003 Jan 15, 75(1):137-145
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Dendritic cell maturation is required for the cross-tolerization of CD8⁺ T cells

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In vivo models have shown that tissue-restricted antigen may be captured by bone marrow-derived cells and cross-presented for the tolerization of CD8⁺ T cells. Although these studies have shown peripheral tolerization of CD8⁺ T cells, the mechanism of antigen transfer and the nature of the antigen-presenting cell (APC) remain undefined. We report here the establishment of an *in vitro* system for the study of cross-tolerance and show that dendritic cells (DCs) phagocytose apoptotic cells and tolerate antigen-specific CD8⁺ T cells when cognate CD4⁺ T helper cells are absent. Using this system, we directly tested the "two-signal" hypothesis for the regulation of priming versus tolerance. We found that the same CD83⁺ myeloid-derived DCs were required for both cross-priming and cross-tolerance. These data suggested that the current model for peripheral T cell tolerance, "signal 1 in the absence of signal 2", requires refinement: the critical checkpoint is not DC maturation, but instead the presence of a third signal, which is active at the DC-CD4⁺ T cell interface.

Although central tolerance offers a mechanism for the deletion of potentially autoreactive cytotoxic T lymphocytes (CTLs), additional strategies must account for the tolerization of T cells specific for tissue-restricted antigen (proteins uniquely expressed in peripheral tissues, for example, β cell-specific antigens¹). Experimental systems used to investigate peripheral tolerance have relied on adoptive transfer of mature naïve CTLs isolated from T cell receptor (TCR)-transgenic mice in which the TCR is specific for peptide epitopes present in host tissue-restricted antigens²⁻⁴. These responding T cells up-regulate activation markers and undergo several rounds of cell division, after which they are tolerized^{2,3}. In addition, bone marrow-derived antigen-presenting cells (APCs), and not the peripheral tissue itself, are responsible for the tolerization of antigen-specific CTLs⁵. This indirect pathway has been termed "cross-tolerance", as exogenous antigen must be cross-presented by the APC, resulting in the generation of major histocompatibility complex (MHC) class I-peptide complexes. Although this data has established a new paradigm for understanding peripheral tolerance, the lack of an *in vitro* system to study cross-tolerance has prevented the precise definition of the cellular events responsible for this *in vivo* phenomenon. Critical unknowns include: (i) the identity of the APC responsible for mediating tolerance; (ii) the mechanism of antigen capture; and (iii) the cellular features that distinguish cross-priming from cross-tolerance.

Using primary human cells, we developed a physiologically relevant system for cross-tolerance that accurately reflects *in vivo* models^{4,5}, thus allowing us to define the cellular mechanism that underlies peripheral tolerance. We found that monocyte-derived DCs phagocytosed apoptotic cells and generated peptide epitopes for presentation on MHC class I molecules. In the absence of CD4⁺ T helper (T_H) cells, the

immunological outcome of cross-presentation was tolerance. Antigen-specific CD8⁺ T cells did not produce interferon- γ (IFN- γ), however they did up-regulate activation markers and undergo cellular proliferation, which was followed by apoptotic death. In the presence of CD4⁺ T_H cells or a stimulus for CD40, which mimicked the cognate CD4⁺ T cell-DC interaction, CD8⁺ antigen-specific T cells produced IFN- γ and developed into effector CTLs. Our data suggested that apoptotic cells serve as a source of antigen, trafficked by DCs to draining lymph organs for the purpose of tolerizing tissue-restricted antigen-specific CD8⁺ T cells.

Using this *in vitro* system to monitor cross-tolerance, we tested the hypothesis that T cell priming versus T cell tolerance is distinguished by the activation state of the APC⁶. We found that DCs with a mature phenotype were required for engagement and tolerization of the CD8⁺ T cells. This finding challenged the current "two-signal" model, which states that the deciding factor in regulation of the immunological outcome of cross-presentation is the expression of costimulatory molecules on mature versus immature DCs^{9,10}. The findings reported here suggested that it is the presence versus absence of CD4⁺ T_H cells that distinguishes "cross-priming" from "cross-tolerance".

Results

CTL activation via the exogenous MHC class I pathway

Human DCs may acquire viral or tumor antigen from apoptotic cells in a manner that permits the formation of MHC class I-peptide complexes and the activation of virus- or tumor-specific CD8⁺ memory T cells, respectively^{11,12}. With a better understanding of the relevant steps involved in the capture and presentation of antigen derived from apoptotic cells^{11,12}, we refined our culturing methodology as follows.

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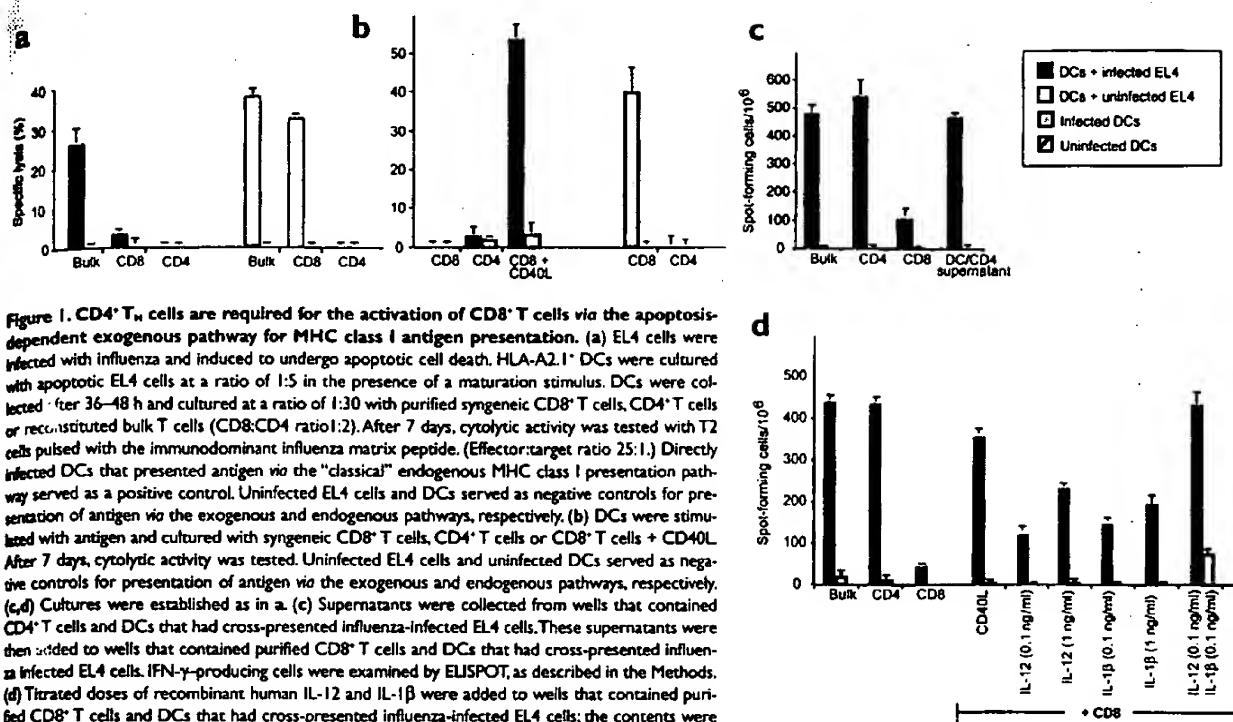


Figure 1. CD4⁺ T_H cells are required for the activation of CD8⁺ T cells via the apoptosis-dependent exogenous pathway for MHC class I antigen presentation. (a) EL4 cells were infected with influenza and induced to undergo apoptotic cell death. HLA-A2.1⁺ DCs were cultured with apoptotic EL4 cells at a ratio of 1:5 in the presence of a maturation stimulus. DCs were collected after 36–48 h and cultured at a ratio of 1:30 with purified syngeneic CD8⁺ T cells, CD4⁺ T cells or reconstituted bulk T cells (CD8:CD4 ratio 1:2). After 7 days, cytolytic activity was tested with T2 cells pulsed with the immunodominant influenza matrix peptide. (Effectortarget ratio 25:1.) Directly infected DCs that presented antigen via the "classical" endogenous MHC class I presentation pathway served as a positive control. Uninfected EL4 cells and DCs served as negative controls for presentation of antigen via the exogenous and endogenous pathways, respectively. (b) DCs were stimulated with antigen and cultured with syngeneic CD8⁺ T cells, CD4⁺ T cells or CD8⁺ T cells + CD40L. After 7 days, cytolytic activity was tested. Uninfected EL4 cells and uninfected DCs served as negative controls for presentation of antigen via the exogenous and endogenous pathways, respectively. (c,d) Cultures were established as in a. (c) Supernatants were collected from wells that contained CD4⁺ T cells and DCs that had cross-presented influenza-infected EL4 cells. These supernatants were then added to wells that contained purified CD8⁺ T cells and DCs that had cross-presented influenza-infected EL4 cells. IFN-γ-producing cells were examined by ELISPOT, as described in the Methods. (d) Titrated doses of recombinant human IL-12 and IL-1β were added to wells that contained purified CD8⁺ T cells and DCs that had cross-presented influenza-infected EL4 cells; the contents were analyzed by ELISPOT. (a–d) Data are representative of >15 experiments; all data are the means ± s.d. of results pooled from triplicate wells. For detection of spot-forming cells (SFCs) see Methods.

Apoptotic cells were cultured with immature DCs in the presence or absence of a maturation stimulus, which mimicked events that occur in the periphery. The DCs were then collected and tested for their ability to activate versus tolerize antigen-specific T cell responses.

To test for the generation of influenza-specific effector cytotoxic T lymphocytes, DCs were first prepared from peripheral blood precursors derived from HLA-A2.1⁺ donors. Uninfected DCs were cultured with influenza-infected apoptotic cells for 36–48 h. The DCs were then collected and tested for their ability to activate a virus-specific, memory CD8⁺ T cell response. Cytotoxicity assays were done with influenza matrix peptide-pulsed targets¹¹. We found that DCs could process exogenous antigen derived from apoptotic cells for the activation of influenza-specific CD8⁺ T cells present in a bulk T cell population (Fig. 1a). When purified CD8⁺ T cells were tested, it was not possible to elicit influenza-specific effector CTLs via the exogenous pathway. In contrast, DCs that were directly infected with influenza, which presented antigen via the endogenous MHC class I antigen-presentation pathway, were capable of activating the purified CD8⁺ T cells in the absence of CD4⁺ T cells (Fig. 1a).

To better define the requirement for T_H cells in antigen cross-presentation, we evaluated strategies for substituting for the CD4⁺ T cells. CD4⁺ T cell–DC engagement is believed to provide CD40 stimulation to the DC^{14–17}. We therefore tested whether CD40 activation might replace the CD4⁺ T cell, thus permitting the activation of CD8⁺ T cells via the exogenous pathway. DCs that were cross-presenting antigen were added to purified CD8⁺ T cells in the presence of CD40 ligand (CD40L) or an agonistic monoclonal antibody (mAb) to CD40; this bypassed the requirement for CD4⁺ T cell help and rendered the DCs capable of activating influenza-specific CD8⁺ T cells (Fig. 1b). Although CD40 ligation was sufficient to substitute for the CD4⁺

cells, it is likely that other pathways also participate, as it was not possible to inhibit CD4⁺ T cells from providing cognate help by treating cultures with CD40-blocking antibodies¹⁴ (data not shown).

We next tested whether cognate help could be substituted by supernatants isolated from cultures that contained purified CD4⁺ T cells and DCs that had cross-presented influenza-infected, apoptotic EL4 cells. This supernatant allowed the activation of influenza-specific CD8⁺ T cells (Fig. 1c). We attempted to detect tumor necrosis factor-α (TNF-α) and interleukin 12 p70 (IL-12p70) in these supernatants. In both cases, cytokine concentrations were below the limit of detection by enzyme-linked immunosorbent assay (ELISA) assay (see Web Tables 1 and 2 on the supplementary information page of *Nature Immunology* online).

Although we could not detect measurable concentrations of cytokines in the supernatants from CD4⁺ T cell–DC cultures, we directly tested whether exogenous recombinant cytokines substituted for the lack of CD4⁺ T cell help. We found that the addition of IL-1β or IL-12 induced the release of IFN-γ by influenza-specific CD8⁺ T cells (Fig. 1d). These cytokines worked additively to maximize activation of the antigen-specific T cells, as was evident from the increased number of influenza-specific IFN-γ-producing cells observed (Fig. 1d). In addition, at the cytokine concentrations used for these experiments, minimal nonspecific T cell activation was observed (Fig. 1d). Although the concentrations of IL-1β and IL-12 required in our *in vitro* studies were nonphysiological, it remains possible that in the microenvironment of the lymph node, the low concentrations of cytokine produced by CD4⁺ T_H cells would be sufficient to achieve antigen-specific cross-priming of CD8⁺ T cells. However, we favored an alternative hypothesis: CD40 ligation of the mature DC acted via an as yet undefined mechanisms to enable DCs to activate CD8⁺ T cells via this exogenous MHC class I

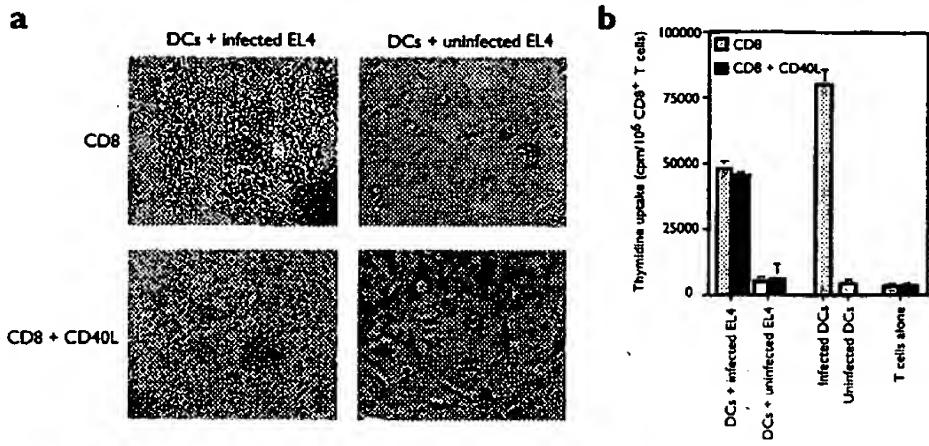


Figure 2. CD8⁺ T cells stimulate exogenous MHC class under CD4⁺ T cell help. (a) Immature DCs were cultured with influenza-infected apoptotic EL4 cells in the presence of CD4⁺ T cell help. (b) Cells were then incubated in the presence of [³H]thymidine for 16 h and proliferation analyzed. Influenza-infected DCs served as positive control. T cells alone served as a control for background [³H]thymidine incorporation. Representative of >5 experiments are the means \pm s.d. of results from triplicate wells.

antigen-presentation pathway. Taken together, our data showed a T_H cell requirement for the production of IFN- γ and the generation of effector CTLs via the exogenous pathway that could be bypassed by CD40 cross-linking and inflammatory cytokines.

Proliferation and tolerization of CD8⁺ T cells

Although the CD8⁺ T cells did not become effector CTLs in response to DCs that cross-presented influenza-infected apoptotic cells (Fig. 1), we did detect evidence of antigen-dependent proliferation during the 7 days of culture (Fig. 2a). This proliferative response was quantified by measuring [³H]thymidine incorporation; we found that the cellular proliferation detected in cultures that contained purified CD8⁺ T cells versus those exposed to DCs in presence of CD40L were equivalent (Fig. 2b). In contrast, when uninfected apoptotic cells were used in the cultures, no T cell proliferation was detected (Fig. 2).

The occurrence of antigen-specific CD8⁺ T cell proliferation in the absence of cytolytic effector function raised the possibility that the proliferating cells were being tolerized, that is, undergoing anergy or deletion¹¹. To directly test this possibility, we established an assay that monitored the three outcomes of antigen cross-presentation: ignorance, activation and tolerance. We also used this system to evaluate the cellular requirements for cross-tolerance. We directly tested the hypothesis that resting APCs (for example, immature DCs) induce tolerance, whereas activated APCs (for example, mature DCs) up-regulate costimulatory molecules and activate CD8⁺ T cells^{9,19,20}.

As described above, DCs were cultured with influenza-infected apoptotic cells for 36–48 h. Either granulocyte monocyte-colony stimulating factor (GM-CSF) + IL-4 or prostaglandin E₂ (PGE₂) + TNF- α were added to the cultures to maintain an immature phenotype or generate mature DC populations, respectively (Fig. 3a). Additionally, macrophages were used as APCs to assess their ability to activate versus tolerize CD8⁺ T cells via the exogenous pathway. Upon collecting the APCs, their phenotype was confirmed by fluorescence-activated cell sorting (FACS). CD14 surface expression was used to identify macrophages. Immature DCs were characterized by their lack of CD14 and CD83 surface expression and low amounts of the costimulatory molecule CD86 (also known as B7-2)²¹. Surface expression of CD83 and CD86 confirmed the generation of mature DC populations (Fig. 3b).

Initially, APCs were assessed for their ability to trigger T cell activation, as measured by IFN- γ production with the use of the enzyme-linked immunospot (ELISPOT) assay. Although this allowed us to eval-

uate the ability of the respective APCs to activate T cells, the response could not distinguish immunological ignorance from tolerization. Therefore, parallel cultures were incubated for 7 days after which time T cells were collected, counted and tested for the presence of influenza-reactive T cells. This was measured by a proliferation assay: autologous influenza-infected DCs were used to maximal activation, thus revealing the presence of any antigen-specific CD8⁺ T cells that remained in the cultures after exposure to those that were cross-presenting influenza antigen (Fig. 3a). If the reactive T cells were actively tolerized, at day 7 the influenza-reactive T cells would not produce IFN- γ . In the event of immunological ignorance, influenza-reactive T cells would persist. In this latter precursor frequency of antigen-reactive CD8⁺ T cells should be alert to the frequency observed at the start of the experiment.

When mature DCs that were cross-presenting antigen were exposed to CD8⁺ T cells in the absence of T_H cells, CD8⁺ T cells were activated and produced only baseline amounts of IFN- γ (Fig. 3c). We determined whether these T cells were ignorant or actively tolerized (Fig. 3a). If ignorant, the influenza-responsive cells would produce IFN- γ ; whereas if tolerized, the influenza-specific T cells would be actively "shut-off" and unable to be recalled. After 7 days of culture, T cells were collected, counted and plated in fresh wells with autologous influenza-infected DCs at a ratio of 30:1. This re-stimulation led to maximal activation of influenza-specific cells present in the culture and provided an assay with which to monitor the immunological outcome that resulted from initial exposure of APCs that were cross-presenting apoptotic cells. In three experiments, no IFN- γ production was detected in the population of CD8⁺ T cells that had been exposed to mature DCs that were cross-presenting influenza antigen (Fig. 3d). We therefore concluded that the influenza-specific T cells had been actively tolerized. A control cell activation included exposing CD8⁺ T cells to DCs that were cross-presenting influenza-infected apoptotic cells in the presence of a specific mAb to CD40. In agreement with published data¹⁴, the addition of CD40 on DCs facilitated T cell activation equivalent to that achieved with DCs that were presenting antigen via the endogenous pathway (Fig. 3c).

When uninfected EL4 cells were used as a source of material, the CD8⁺ T cells did not proliferate (Fig. 2b and 3a, shown); in addition, when the T cells were removed from the

stimulated via the CD40 pathway in the absence of CD40L. After 36–48 h, DCs were treated with syngeneic CD40L. After 3 days, cultures were assayed for the presence of 4 µg/ml T cell proliferation. Infected DCs and T cells alone were used as background controls. Data are from three independent experiments; all data are pooled from

the lack of a T cell response from active DCs for 7 days, for the presence of a T cell response to a restimulated T cell to provide immunological ignorance to DCs during the antigen-specific recognition. In the antigen-specific recognition, the T cell response would be equivalent.

Cells exposed to DCs were not activated (Fig. 3c). We next examined whether DCs could persist, as they are the APCs and cells would be exposed to the same three populations of T cells. The cross-presenting T cells were able to maintain their engagement with the T cells, even though they were not exposed to the antigen-specific T cells. This indicated that the T cells were cross-presented by the DCs.

Use of immature DCs as APCs also resulted in an immunological outcome of ignorance. Although immature DCs are capable of cross-presenting antigen and generating surface MHC class I-peptide complexes¹³, they were not able to stimulate IFN-γ production, even in the presence of agonistic mAb to CD40 (Fig. 3c). CD40 stimulation of

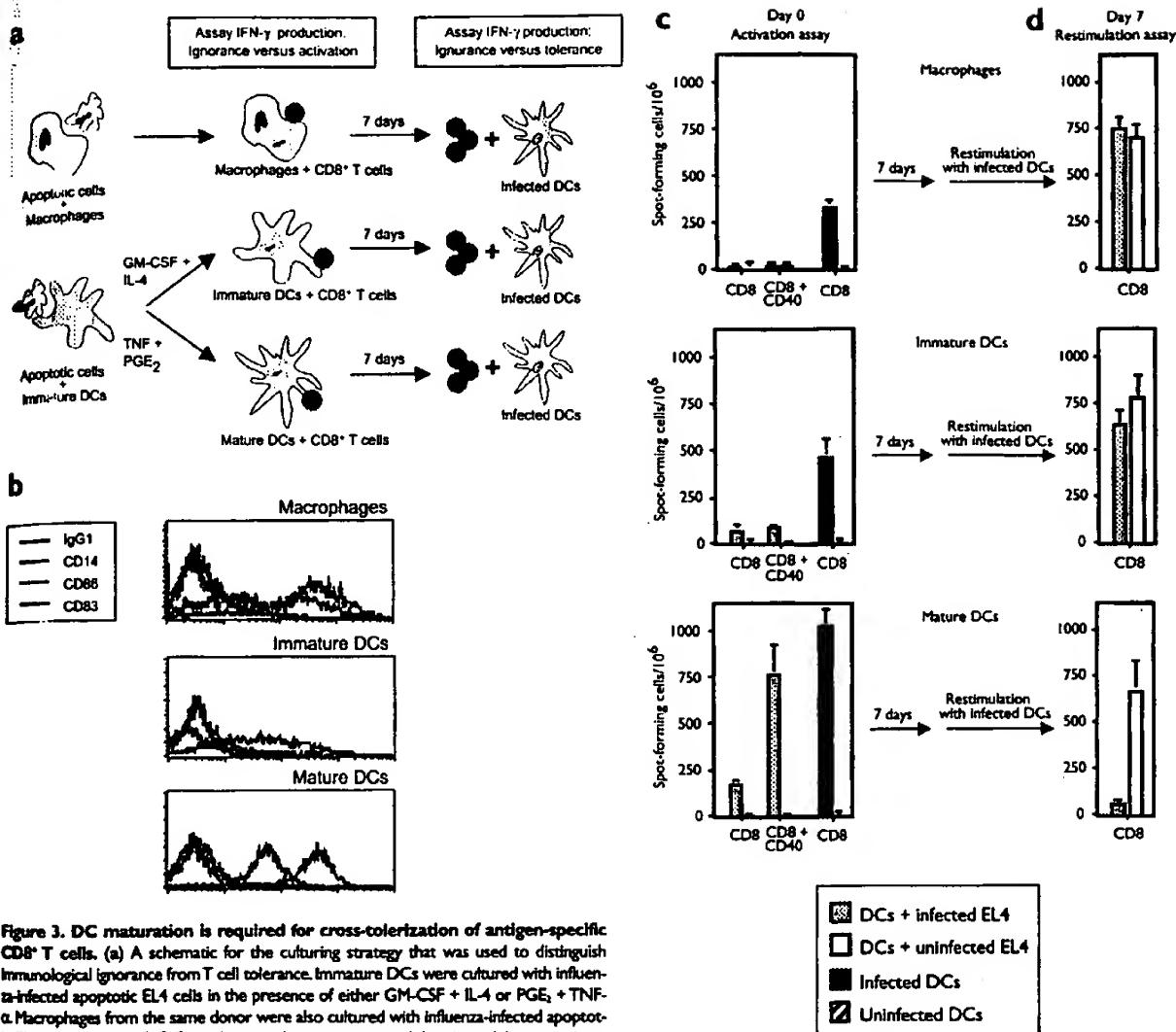


Figure 3. DC maturation is required for cross-tolerization of antigen-specific CD8⁺ T cells. (a) A schematic for the culturing strategy that was used to distinguish immunological ignorance from T cell tolerance. Immature DCs were cultured with influenza-infected apoptotic EL4 cells in the presence of either GM-CSF + IL-4 or PGE₂ + TNF-_α. Macrophages from the same donor were also cultured with influenza-infected apoptotic EL4 cells. At time 0, DCs and macrophages were tested for their ability to activate influenza-specific T cells via the exogenous pathway. Parallel cultures were tested to distinguish the other two potential outcomes of antigen cross-presentation: ignorance versus tolerance. Restimulation of the remaining T cells on day 7 established the persistence of influenza-reactive T cells after exposure to APCs that were cross-presenting antigen. If no IFN-_γ production was detected, it would suggest tolerization of the influenza-reactive T cells during the 7 day culture period. (b) Upon collecting the APCs after 36 h of culture, the cellular phenotype was confirmed by FACS analysis. (c) After capture of the apoptotic EL4 cells, the DC and macrophage populations were cultured with syngeneic CD8⁺ T cells to assess IFN-_γ production. APCs were placed in an ELISPOT well with purified CD8⁺ T cells with or without an agonistic CD40 mAb. (d) After 7 days of culture, T cells were collected and cells that excluded trypan blue were counted and placed in fresh wells with autologous influenza-infected DCs. Data are representative of three experiments.

and restimulated, influenza-reactive CD8⁺ T cells were detected (Fig. 3d). This data showed that in the absence of antigen, the influenza-specific CD8⁺ T cells remained immunologically ignorant during the 7 days of culture. This interpretation was strengthened by the observation that the precursor frequency of influenza-specific CD8⁺ T cells (~0.1% in this experiment) was unchanged during the 7-day culture; if nonspecific T cell death had occurred, it did not bias the antigen-specificity of the cultures.

Use of immature DCs as APCs also resulted in an immunological outcome of ignorance. Although immature DCs are capable of cross-presenting antigen and generating surface MHC class I-peptide complexes¹³, they were not able to stimulate IFN-_γ production, even in the presence of agonistic mAb to CD40 (Fig. 3c). CD40 stimulation of

immature DCs was not sufficient to permit T cell activation; this may be due to low surface expression of costimulatory molecules on immature DCs (Fig. 3b) or the result of low CD40 expression in comparison to expression on mature DCs (data not shown). When parallel cultures were restimulated with autologous influenza-infected DCs, the precursor frequency of influenza-specific CD8⁺ T cells was equivalent to that at the start of the culturing period, even when influenza-infected apoptotic cells were used in the assays (Fig. 3d).

Macrophages were also unable to stimulate IFN-_γ production, even in the presence of agonistic mAb to CD40 (Fig. 3c). Again, as shown by the restimulation assay, under these conditions, the CD8⁺ T cells remained immunologically ignorant (Fig. 3d). This was consistent with the fact that macrophages are not capable of cross-presenting antigen¹².

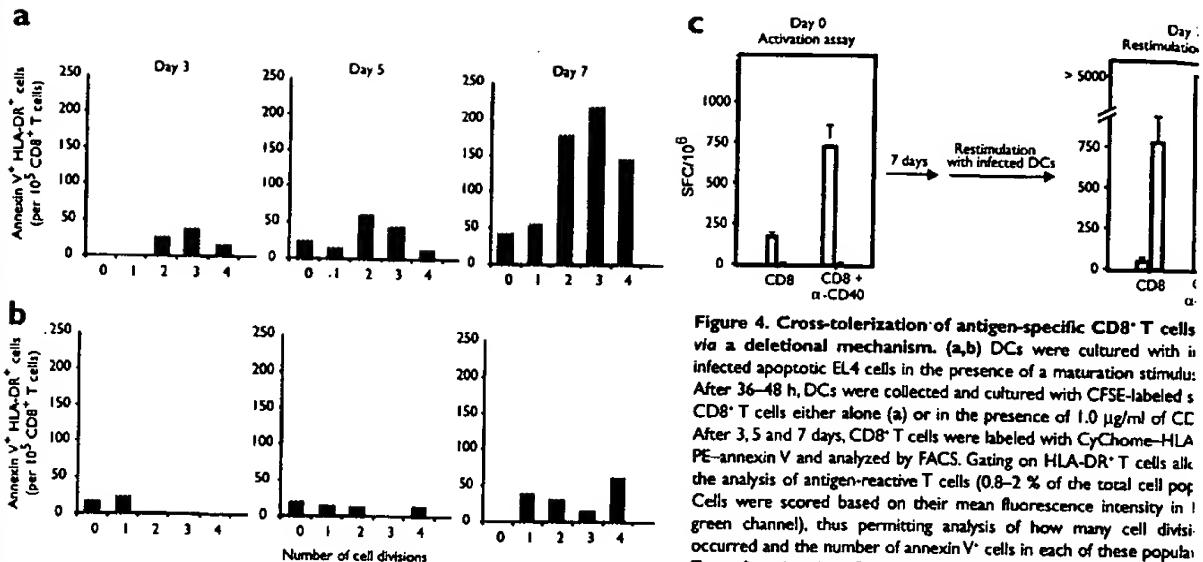


Figure 4. Cross-tolerization of antigen-specific CD8⁺ T cells via a deletional mechanism. (a,b) DCs were cultured with infected apoptotic EL4 cells in the presence of a maturation stimulus. After 36–48 h, DCs were collected and cultured with CFSE-labeled sCD8⁺ T cells either alone (a) or in the presence of 1.0 μ g/ml of CC. After 3, 5 and 7 days, CD8⁺ T cells were labeled with CyChome-HLA PE-annexin V and analyzed by FACS. Gating on HLA-DR⁺ T cells alk the analysis of antigen-reactive T cells (0.8–2% of the total cell pop). Cells were scored based on their mean fluorescence intensity in green channel), thus permitting analysis of how many cell divisions occurred and the number of annexin V⁺ cells in each of these populations. To confirm that the influenza-reactive CD8⁺ T cells were deleted, a proliferation assay was done on the remaining T cells. After 7 days of culture, the proliferation of CD8⁺ T cells was measured in the presence of CD40L were tested.

were collected and plated in fresh wells. As a control for T cell activation, CD8⁺ T cells exposed to DCs cross-presenting antigen in the presence of CD40L were tested. Filled bars, DCs that were cross-presenting influenza-infected EL4 cells; open bars, DCs that were cross-presenting uninfected EL4 cells.

To show the integrity of each cell type, we also examined the ability of each APC population to activate T cells via the endogenous MHC class I presentation pathway. We found that when antigen-loading occurred via the "classical" MHC class I pathway, the macrophages and immature DCs were able to trigger antigen-specific IFN- γ release in the CD8⁺ T cells (Fig. 3c).

Cross-tolerization of CD8⁺ T cells

We next attempted to define the mechanism of T cell tolerance after antigen cross-presentation by following the fate of the dividing antigen-reactive T cells (Fig. 2b). CD8⁺ T cells were labeled with the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) and cultured for 7 days with DCs that had phagocytosed influenza-infected apoptotic EL4 cells. After 3, 5 and 7 days of culture, samples were labeled for HLA-DR, a marker for T cell activation in humans. In addition, annexin V was used, which tests for the exposure of phosphatidylserine on the outer leaflet of the plasma membrane, a marker for early apoptotic death. With the use of FACS, the HLA-DR⁺ T cells were gated and CFSE fluorescence and annexin V staining simultaneously evaluated.

On day 3, ~10% of the HLA-DR⁺, CD8⁺ T cells had divided and initiated an apoptotic pathway. On day 5, >35% of the dividing HLA-DR⁺ CD8⁺ T cells were annexin V⁺; by day 7, >50% of the proliferating HLA-DR⁺ CD8⁺ T cells were committed to die (Fig. 4a). By analyzing the relative CFSE intensity, we determined that most antigen-reactive cells divided two to four times before initiating a programmed cell death (Fig. 4a). The HLA-DR⁺ CD8⁺ T cells represented <2% of the T cell population and, in contrast to the death observed in this sub-population of antigen-reactive T cells, <5% of annexin V staining was detected in the HLA-DR⁺ nonproliferating T cells (data not shown).

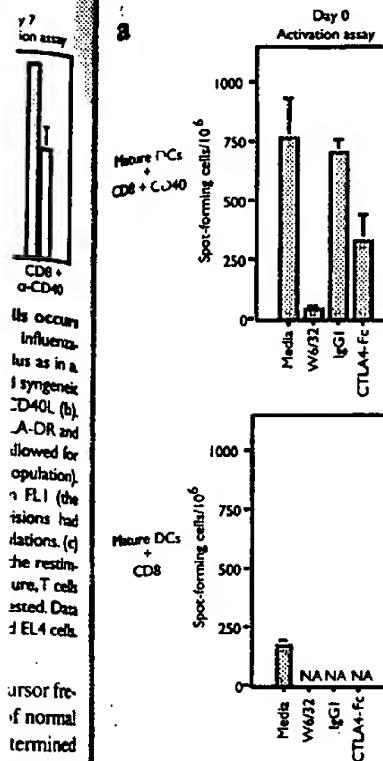
In CD8⁺ T cell-DC cultures exposed to a CD40 stimulus, equivalent numbers of dividing HLA-DR⁺ cells could be detected, however, low numbers of cells were committed to apoptosis. Even at day 7, <6% of the proliferating HLA-DR⁺ CD8⁺ T cells were annexin V⁺ (Fig. 4b). Again, these responding T cells represented a small fraction of the

overall population, which was consistent with the defined precursor frequencies of influenza-specific T cells in the peripheral blood of volunteers (0.01–2.0% influenza-specific precursors, range determined in a screen of >100 blood donors). The lack of apoptosis in the cultures was in agreement with the observation that CD40 engagement by DCs facilitated the generation of effector CTLs (Fig. 1). This was confirmed with the restimulation assay described above. In the presence of CD40, the expanded population of CD8⁺ T cells could be reactivated, which resulted in a precursor frequency seven-times that observed at the start of the culturing period (Fig. 4c). We therefore conclude that in the presence of a CD40 stimulus, the CD8⁺ T cells did not undergo cell death because the DCs had been converted from a "tolerizing" to an "activating" phenotype.

These data indicated that we had identified an *in vitro* strategy for monitoring the cross-tolerization of antigen-reactive CD8⁺ T cells. T cells that engaged a DC cross-presenting antigen in the absence of CD40, divided and were subsequently deleted. Based on *in vivo* results, it had been assumed that the CD8⁺ T cell proliferation that occurred before tolerization constituted transient activation and that the subsequent death of the antigen-specific cells was analogous to active induced cell death¹¹. We show, however, that although the antigen-reactive cells divided and expressed "activation markers", they did not produce IFN- γ nor did they become effector CTLs, thus, they should not be considered "activated". Although T cell tolerance is an active process, it seems to act upstream of T cell effector function.

Role of costimulatory molecules in cross-tolerance

The current "two signal" model for T cell activation *versus* tolerance proposes that in the absence of costimulatory molecular interaction (such as CD80 and CD86), TCR engagement results in tolerance^{22,23}. According to this model, a maturation stimulus for immature DCs, possibly offered by a "danger signal", is what distinguishes tolerance *versus* activation^{19,24}. To further test this hypothesis and strengthen our assertion that DC maturation is required for tolerization of CD8⁺ T cells via the exogenous pathway, we examined the effect of inhi-



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engagement of either the TCR ("signal 1") or CD28 (one of the dominant "signal 2" receptors) on T cells.

CD8+ T cells were exposed to antigen-charged mature DCs in the presence of W6/32 (a blocking mAb specific for HLA-A, HLA-B and HLA-C) or cytotoxic T lymphocyte antigen-4-Fc fusion protein (CTLA4-Fc, which binds B7-1 and B7-2, thus blocking engagement of CD28). In the presence of W6/32, T cell activation was completely inhibited (Fig. 5a), as was proliferation at day 4.5 (data not shown). Parallel cultures were incubated for 7 days and the T cells were collected and tested in the restimulation assay. The presence of an inhibitor of MHC class I-TCR engagement effectively blocked the tolerization of T cells, as was evident from the restimulation of an influenza-specific immune response after 7 days of culture (Fig. 5b). Thus, without engagement of "signal 1", the T cells were neither activated nor tolerized. This showed that the tolerizing phenotype was dependent on a cognate interaction between the DC and the CD8+ T cell.

Inhibition of "signal 2" by CTLA4-Fc gave a partial phenotype in three independent experiments: 45–60% inhibition of T cell activation (Fig. 5a); 30–50% inhibition of proliferation at day 4.5 (data not shown); and 40–50% inhibition of tolerance induction (Fig. 5b). Although we measured a reproducible inhibition of tolerance with CTLA4-Fc, these data suggested that multiple costimulatory molecules are important for efficient tolerization of antigen-specific CD8+ T cells. This finding was consistent with the finding that mature DCs up-regulated several molecules that are important for engagement with T cells, including intercellular adhesion molecule 1 (ICAM-1 or CD54), heat stable antigen (HSA or CD24) and lymphocyte function-associated antigen-3 (LFA-3 or CD58).

Figure 5. Costimulatory molecules are important for the cross-tolerization of CD8+ T cells. (a) To test the role of MHC class I-TCR and B7-CD28 engagement in cross-tolerization, CD8+ T cells were exposed to mature DCs, which had cross-presented influenza antigen, in the presence of W6/32, a control IgG1 antibody or CTLA4-Fc. Cultures were tested on day 0 in the presence of an agonistic CD40 mAb to determine the effect of these blocking agents on T cell activation. They were also tested on day 7 in the absence of CD40 stimulus to determine the effect on cross-tolerance. W6/32 inhibited T cell activation by 95% (a) and completely abrogated the ability to tolerate influenza-specific CD8+ T cells (b). CTLA4-Fc inhibited T cell activation by 60% (a) and tolerance by ~40% in the experiment shown (b). Data are representative of three experiments; all data are the means \pm s.d. of pooled results. NA, not applicable; filled bars, DCs that were cross-presenting influenza-infected EL4 cells. DCs that were cross-presenting uninfected EL4 cells are not shown.

Discussion

Apoptotic death itself is not an endpoint: dying cells are capable of transferring antigen to the immune system. Apoptosis is recognized as the primary mechanism whereby physiological cell death occurs both in quiescent tissue (for example, during normal cell turnover) and at sites of inflammation. DCs exist in peripheral tissues where they capture these dying cells²³. Rather than degrading the internalized material, as is the case for macrophages, the DC processes antigen derived from the apoptotic cell and generates peptide epitopes for presentation on MHC class I¹. This pathway is referred to as cross-presentation for its ability to "cross" classically defined restrictions for MHC class I antigen presentation²⁴. Here we used a model system for antigen cross-presentation^{11,12} to investigate a possible mechanism by which tissue-restricted proteins may access the DC for purposes of tolerizing antigen-specific CD8+ T cells.

Our data suggested that the bone marrow-derived cell responsible for mediating the *in vivo* phenomenon of cross-tolerance was the DC and that antigen transfer may have been achieved by phagocytosis of apoptotic material, thus permitting access to MHC class I molecules. DCs are responsible for the cross-presentation of antigen²⁵, however, our findings provide direct evidence that DCs also mediate T cell tolerance. In addition, we have shown that CD4+ T cells dictate the immunological outcome of cross-presentation: in their absence, the CD8+ T cells divided and were subsequently tolerized via a deletion mechanism. One important caveat to these experiments is that our system relied on a previously expanded population of influenza-specific memory T cells. Although we were unable to directly address experimental questions with naïve T cells in humans, we believe that our ability to tolerate memory T cell responses shows the potency of this apoptosis-dependent cross-tolerizing pathway.

An unexpected result borne from our studies challenges a major paradigm in the field of immunobiology. We showed that to achieve cross-tolerance, DC maturation was required. The critical checkpoint does not appear to be a maturation stimulus—as suggested by the "two signal" hypothesis—instead it is the presence of CD4+ T_H cells, which act in part by delivering a signal to the mature DC via CD40. At first, our findings appeared to conflict with the results from *in vivo* studies; in these studies, injection of activating anti-CD40 into mice primed T cells that, under quiescent conditions, were tolerized^{20,21}. From these data it was concluded that CD40 stimulation matured the DCs, thus facilitating T cell stimulation²⁰. However, our data suggest an alternative interpretation of these results: CD40 provided a third signal to the DCs, thus bypassing the need for CD4+ T_H cells in the cross-priming of antigen-specific T cells. Although engagement of CD40 on immature DCs can indeed result in maturation, this was not the role it played in our studies, and arguably, it was not what was occurring *in vivo*.

Physiologically, DCs must migrate to the T cell-rich areas of the lymph node before engaging a CD4⁺ T_H cell, the proposed source of the CD40 stimulus. In light of this model, the requirements for DCs to reach the T cell zone of draining lymph organs must be reassessed. Only mature DCs seem capable of accessing such areas, as expression of the chemokine receptor CCR7 (expressed on mature but not immature DCs) is critical for T cell-DC colocalization^{10,11}. Although this does not preclude a role for immature DCs in skewing T cell responses¹², it suggests that the migratory DCs, which traffic the apoptotic material from the periphery, have the potential to directly tolerize self-reactive CD8⁺ T cells.

Two significant unknowns remain: how do DCs undergo maturation *in vivo* and what does CD40L do to influence the immunological outcome of antigen cross-presentation? With respect to the DC differentiating from an immature to a mature phenotype, one possibility is that phagocytosis followed by reverse transmigration (adluminal-to-luminal) provides the requisite maturation stimulus¹³. Irrespective of the source, our data suggested that the presence of a maturation stimulus regulates immunological ignorance *versus* immunity but does not determine the nature of that immune response. Once in the draining lymph organ, the presence *versus* absence of T_H cells interacting with the mature DC distinguish the priming *versus* tolerization of antigen-specific CD8⁺ T cells.

In considering the role of CD40L-CD40 engagement in the CD4⁺ T_H cell-DC interaction, a number of possibilities warrant consideration. Notably, CD40L triggers DC maturation, increases survival of the DC and promotes up-regulation of the transporter associated with antigen processing (TAP) expression¹⁴⁻¹⁶. The DCs exposed to CD40L were already of a mature CD83⁺HLA-DR^{hi} phenotype, and we confirmed that CD40L treatment did not result in enhanced expression of known markers for DC maturation (see Web Fig. 1 on the supplementary information page of *Nature Immunology* online and unpublished data). Regarding increased DC survival, the activation of CD40 may result in more DCs, thus facilitating activation of antigen-specific T cells. This explanation would not account for the ELISPOT results obtained at 40 h, as no difference in viability was noted between CD40L-treated *versus* untreated groups until 72 h¹³ and data not shown). In considering the up-regulation of TAP by CD40L exposure, we analyzed the expression of MHC class I on the surface of mature DCs before and after CD40 ligation (Web Fig. 2). Although there was no measurable difference in surface MHC class I, it was not possible to establish the density of the relevant influenza MHC class I-peptide complexes. It therefore remains a possibility that increased production and/or stability of cognate MHC class I-peptide complexes on CD40L-treated DCs was responsible for converting the "tolerizing" DC to an "activating" DC.

Further experimental work will be required to resolve what we have identified as the two possible models for distinguishing cross-tolerance from cross-priming: "The peptide rheostat model" *versus* "The molecular switch hypothesis." The former model argues that the surface MHC class I-peptide density would distinguish priming from tolerance (Web Fig. 3a). Based on the data presented here and on data that shows the sensitivity of the cross-presentation pathway to the pharmacological agent FK506 (M. Jegathesan *et al.*, unpublished data), we favor the latter model. We propose that CD40 receptor signaling activates a critical regulatory switch in DCs that is responsible for revealing a third signal; this, in turn, results in the activation of T cells *via* the exogenous MHC class I antigen-presentation pathway (see Web Fig. 3b). We suggest that the current model for peripheral tolerance, "signal 1 in the absence of signal 2", requires refinement; the critical checkpoint is not

the expression of costimulatory molecules triggered by DC maturation but instead is the presence of CD4⁺ T cells, which act on a mature DC.

Methods

Isolation and preparation of cell. Peripheral blood mononuclear cells (PBMCs), DCs, macrophages and T cells were prepared as described¹⁷. Briefly, peripheral blood was obtained from normal donors in heparinized syringes and PBMCs were isolated by sedimentation over Ficoll-Hypaque (Pharmacia Biotech, Piscataway, NJ). T cell-enriched and T cell-depleted fractions were prepared by rosetting with neuraminidase-treated sheep red blood cells. Immature DCs were prepared from the T cell-depleted fraction by culturing cells in the presence of GM-CSF (Immunex, Seattle, WA) and IL-4 (R&D Systems, Minneapolis, MN) for 7 days. GM-CSF (1000 U/ml) and IL-4 (500 U/ml) were added to the cultures on days 0, 2 and 4. To generate mature DCs, the cultures were transferred to fresh wells on days 6-7 and monocyte-conditioned media (MCM)¹⁸ or a mixture of 50 U of TNF- α (Endogen, Boston, MA) and 0.1 μ M of PGE₂ (Sigma, St. Louis, MO) were added for an additional 1-2 days. At days 6-7, >95% of the cells were CD14⁺CD83⁺HLA-DR^{hi} DCs. After maturation, on days 8-9, 70-95% of the cells were of the mature CD14⁺CD83⁺HLA-DR^{hi} phenotype. Use of PGE₂ did not alter the ability to use the mature DCs to elicit a T_H1 immune response equivalent to that observed with MCM¹⁹. CD4⁺ and CD8⁺ T cells were further purified to >99% purity by positive selection with the MACS column purification system (Miltenyi Biotech, Auburn, CA).

Induction of apoptotic death. The mouse lymphoma cell line EL4 (number TIB-39, ATCC, Rockville, MD) was used as a source of apoptotic cells because they can be efficiently infected with influenza virus and do not induce marked background T cell activation to mouse antigens. The EL4 cells were infected with influenza and apoptosis was triggered using a 60W lamp (Derma Control Inc., Old Forge, PA), calibrated to provide 2 mJ/cm²/s. These cells undergo early apoptotic death within 8-10 h. Cell death was confirmed with the Ea Apoptosis Detection Kit (Kaiyima Biomedical, Seattle, WA). To ensure that we were staining the uptake of early apoptotic cells, the kinetics of death were carefully worked out: 10 hours after irradiation, EL4 cells first externalize phosphatidylserine on the outer leaflet of their cell membrane, as detected with annexin V. By 10-16 h, these cells were TUNEL⁺. It was not until 36-48 h later that the majority of cells included trypan blue in the cytoplasm, an indicator of secondary necrosis (data not shown).

Detection of antigen-specific T cells: ELISPOT assay for IFN- γ release. Immature DC apoptotic cells and a DC maturation stimulus (MCM, or a combination of TNF- α + PGE₂) were incubated together for 36-48 h to allow phagocytosis of the apoptotic EL4 cells, antigen processing and DC maturation to occur. The DCs were collected, counted and added to purified T cell populations in plates that had been precoated with 10 μ g/ml of a prim IFN- γ mAb (clone Mab-1-DIK, Mabtech, Cincinnati, OH). In all experiments, 2 \times 10⁴ T cells were added to 6.67 \times 10³ DCs to give a 30:1 DC:T cell ratio. The cultures were incubated 40-44 h at 37 °C. Cells were washed out with mild detergent and the well contents incubated with 1 μ g/ml of a biotin-conjugated IFN- γ mAb (clone Mab 7BG-1, Mabtech). Well contents were stained with the Vectastain Elite kit, as per manufacturers instructions (Vec Laboratories, Burlingame, CA). Colored spots represented the IFN- γ -releasing cells and were reported as spot forming cells/10⁴ cells. Data from triplicate wells were pooled and mean data reported.

Detection of antigen-specific T cells: Proliferation assay. Influenza-infected or uninfected apoptotic cells were cultured with 2 \times 10⁴ purified T cells and DCs. Cultures were established as described above. After 4.5 days, cultures were pulsed with 4 μ Ci/ml [³H]thymidine and collected 16 h later.

Detection of antigen-specific T cells: ⁵¹Cr release assay. PBMCs derived from HLA-A2.1⁺ individuals were used for the detection of influenza-specific effector T cells. Influenza-infected apoptotic EL4 cells were cultured with immature DCs in the presence of a maturation stimulus for 36-48 h before the establishment of T cell-DC cultures¹⁹. After 3 days the cytolytic activity of responding T cells was analyzed with the use of T2 cells (TAP-deficient HLA-A2.1⁺ cell line) that had been pulsed for 1 h with 1 μ M of the immunodominant influenza matrix peptide, GILGFVFTL²⁰. Specific lysis = (% killing of T2 cells - (% killing of T2 cells alone)). Background lysis ranged between 0-16%. Influenza-infected DCs served as a positive control in all experiments.

Detection of proliferating T cells that were dying *via* apoptosis. T cells were labeled with 0.1 μ M CFSE and cultured for 7 days with DCs. CFSE-labeled cells divide and daughter cells receive approximately half the fluorescent dye, thus allowing for the monitoring of proliferation through four to five rounds of cell division. In studying natural immune responses in humans, we were limited by the low precursor frequencies of antigen-specific T cells (0.02-1.2% influenza-specific precursors), as compared to studies that use TCR-transgenic mice. Thus, to assess cell death in the antigen-responsive cells, we labeled the T cell populations with an HLA-DR mAb. Compared to other activation markers (such as CD25, CD38, CD69) HLA-DR expression showed the lowest background labeling in unstimulated T cells (data not shown). With the use of annexin V, T cells were evaluated for exposure of phosphatidylserine on the outer leaflet of their plasma membrane, an indicator of early apoptosis. After 3, 5 or 7 days, T cells were removed from the cultures and labeled with CyChrome-HLA-DR mAb (PharMingen, San Diego, CA) and phycoerythrin (PE)-annexin

maturity, mature DCs

²BMCs), DCs, rat blood was isolated by sedi- l-enriched andated sheep red in by culturing (D&D Systems, were added to transferred to culture of 50 U/ml O) were added 2B3 HLA-DR if the mature use the mature M¹, CD4⁺ and the MACS col-

(Keyma Biomedical). Samples were analyzed with a FACScan (Becton Dickinson, San Jose, CA) and the HLA-DR⁺ T cells were gated based on forward scatter and fluorescence in FL3. Positive cells were gated and annexin V staining and relative CFSE fluorescence evaluated.

Additional antibodies and reagents. A blocking mAb specific for HLA-A, HLA-B and HLA-C (W6/32) was from BD-PharMingen (San Diego, CA); the agonistic CD40 mAb (clone 52C6) was from Mabtech; CD40L was from Alexis Biochemicals (San Diego, CA); CTLA-4-Fe was from Research Diagnostic (Flanders, NJ).

Phase contrast microscopy. Cultures were examined with a Zeiss Axiovert phase contrast microscope with a $\times 20$ objective (Zeiss, Thornwood, NY).

Note: Supplementary information can be found on the Nature Immunology website (http://immunology.nature.com/suppl_info/).

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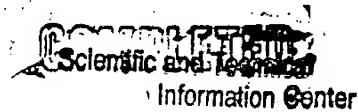
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RECOMMENDATIONS FOR THE IMPLEMENTATION OF NEORAL C₂ MONITORING IN CLINICAL PRACTICE

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INTRODUCTION

The weight of published evidence has demonstrated that patient management by Neoral C₂ monitoring (i.e. adjusting doses according to drug levels at 2 hours postdose), compared with management by conventional "trough" concentration can improve clinical outcomes for de novo renal and liver transplant patients (1-9). Initial studies suggest that Neoral C₂ monitoring also can detect cyclosporine overexposure in maintenance patients. Evidence to date has demonstrated the benefit of dose reduction in these patients, in terms of improving short-term renal function and reducing hypertension (10-11).

The weight of evidence together with the establishment of target C₂ levels for both renal and liver transplantation, and the development of a simple dose-adjustment formula (Fig. 1), have stimulated the implementation of Neoral C₂ monitoring in clinical practice. Neoral C₂ targets have been established for both de novo and maintenance liver transplant patients (Table 1) (12). Target levels for renal transplant patients based on accumulated data to date are outlined in Table 2 (12,13).

As a result of this progress, Neoral C₂ monitoring has now been successfully adopted by transplant centers worldwide, across healthcare systems and patient types. The collective experience of a cross-section of these centers has provided

recommendations for the successful implementation of Neoral C₂ monitoring in clinical practice. These recommendations focus on a step-wise approach to implementation. Three major objectives of this approach are: to ensure the commitment of all unit staff and patients affected by the change to C₂ monitoring; to set up a system that ensures that patients are sampled on time and that C₂ and C₀ samples can be differentiated at all stages, through to reporting; and to minimize the impact of implementation on workload, and clinic and ward routines. The major steps in the implementation process are outlined in Figure 2.

A STEP-WISE APPROACH

Step 1: Review of Clinical Evidence

The first step involves a review of the scientific rationale and clinical evidence by the clinical team, to ensure that everyone is committed to the implementation of this new technique.

Step 2: Presentation of the C₂ Concept

The second—and most critical—step in the process involves the presentation of the scientific rationale for and clinical benefits of Neoral C₂ monitoring to all members of the transplant team, the external departments that will be affected by the change from C₀ to C₂ monitoring (e.g., clinical laboratory staff), and the satellite units that refer patients for transplantation. Without the understanding and agreement of everyone involved, the implementation of Neoral C₂ monitoring is likely to be undermined at a later stage with the potential for patient safety to be compromised and effort wasted. Some units have published a short newsletter to sustain communication with all those involved in the change to Neoral C₂ monitoring, as a follow up to the initial meeting.

At this stage, timelines for implementation and the patient population in which the process will be piloted should also be agreed upon. Centers have found that this is done most easily by focusing first on de novo cases (see Step 5). In addition, contact should be established with referring satellite units to decide what arrangements will be made for sampling patients after discharge.

Step 3: Formation of the Transition Team

The third step involves the formation of a multidisciplinary transition team consisting of members from key departments whose role it will be to drive implementation by:

New Neoral dose = Old Neoral dose \times	Target C ₂ level
Current C ₂ level	

FIGURE 1. Neoral C₂ dose adjustment formula (13).

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- Championing Neoral C₂ monitoring and acting as an information resource within their own departments
- Contributing their expertise to the unit implementation protocol
- Training staff within their own departments

It is important at this stage to appoint an enthusiastic and experienced team leader who will have responsibility for overseeing the whole implementation process, keeping to agreed timelines, and liaising within and between departments.

Step 4: System Development

Once the transition team is formed, an implementation protocol should be developed. Ward, clinic, and laboratory systems will need to be set up to ensure that patients take their dose on time, blood samples are taken 2-hours postdose (± 15 min) (14), and that C₂ and C₀ samples can be differentiated at all stages, through to final reporting. Establishing a protocol is particularly important at the outset because staff will be less familiar with Neoral C₂ sampling. It is intended that the set up of ward, clinic, and laboratory systems should happen concurrently.

Implementation in the Inpatient Setting. The inpatient setting is an ideal starting point for the implementation of Neoral C₂ monitoring because it offers a relatively controlled environment compared to the outpatient clinic. Also, by pi-

TABLE 1. Target Neoral C₂ levels for liver transplant patients (12)

Time posttransplant (months)	Cyclosporine level ($\mu\text{g}/\text{mL}$)
0-3	1.0
4-6	0.8
>6	0.6

Neoral C₂ targets have been established for both de novo and maintenance liver transplant patients (12). Target values should be achieved by day 5 posttransplant for optimal outcome. These targets are valid only for the Neoral formulation of cyclosporine and are based on twice-daily administration. No change in target level is required for different assay types. C₂ samples must be taken at 2 hours ± 15 minutes postdose (14).

TABLE 2. Target Neoral C₂ levels for renal transplant patients (12,13)

Time posttransplant (months)	Cyclosporine level ($\mu\text{g}/\text{mL}$)
1	1.5-2.0
2	1.5
3	1.3
4-6	1.1
7-12	0.9
>12	0.8

Neoral C₂ target levels have been established for the first month posttransplant in adult renal transplant patients (12). Target levels beyond the first month for renal transplant patients are based on accumulated data to date (13). Appropriate target levels beyond the first month will be further refined through ongoing clinical trials. Target values should be achieved by day 5 posttransplant for optimal outcome. These targets are valid only for the Neoral formulation of cyclosporine and are based on twice-daily administration. No change in target level is required for different assay types. C₂ samples must be taken at 2 hours ± 15 minutes postdose (14).

luting procedures with a small number of patients, a great deal can be learned before full-scale implementation in the maintenance population.

Experience from centers that have implemented Neoral C₂ monitoring suggests that all nursing staff should be trained in the benefits and procedures involved with Neoral C₂ monitoring. It is also advisable that one member of the nursing staff oversee the monitoring of patients on each daytime shift in order to streamline communication with patients, the phlebotomy service, the clinical laboratory, and junior medical staff. The appointed nurse has the responsibility for making sure that the patients take their Neoral at the designated time, that the phlebotomist takes the blood samples by the 2-hour cut off point, and that samples are clearly labeled and dispatched to the laboratory. In some centers it may be necessary for dosing and sampling of Neoral C₂ patients to be staggered, depending on the capacity of the phlebotomy service and the needs of individual patients.

A documentation system for Neoral C₂ patients is essential to the efficiency of this process to minimize the workload of the ward staff and phlebotomists. The documentation system could consist of the following elements, adapted for local use:

- Quick identification of Neoral C₂ patients by means of a

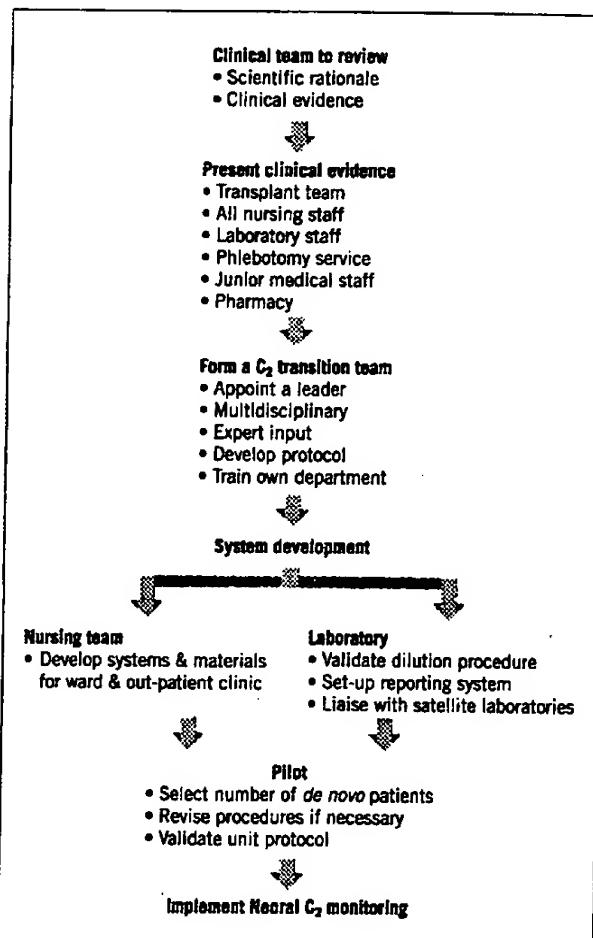


FIGURE 2. Step-wise process for implementation of Neoral C₂ monitoring.

clearly visible list (e.g. a wall chart) and readily available Neoral C₂ labels to be adhered to the patients' notes

- Documentation of dosing and sampling times and Neoral C₂ levels on specific C₂ inserts for the patients' notes
- A checklist of Neoral C₂ patients to ensure patients are not overlooked
- Readily available Neoral C₂ labels to be adhered to the blood samples and to the packaging for dispatch

The system for differentiating between C₂ and C₀ samples on the ward can be further enhanced by separately timing phlebotomy for these two groups. For patients being monitored by C₀, all blood work can be scheduled for early morning (e.g. 8:30 AM) when Neoral C₂ patients are administered their dose of Neoral by the designated nurse. The phlebotomist then returns 2 hours later (e.g. 10:30 AM) to perform all the blood work for the Neoral C₂ patients, including the C₂ sample, and to label the C₂ samples for dispatch to the laboratory by the agreed time. Separate timing prevents mixing up C₀ and C₂ samples and enables both sets of patients to complete all their blood work at once. In the event that a patient's blood sample is taken late, the time should be noted, the laboratory informed, and sampling rescheduled for the next day.

The ideal opportunity to educate patients about this new management technique is while they are on the ward. The designated nurse, or a clinician or pharmacist, should educate patients about the outcome benefits of Neoral C₂ monitoring and the need to have their blood sample taken 2 hours (\pm 15 min) postdose. Harnessing the enthusiasm of patients and promoting their understanding of the benefits of tailoring their Neoral therapy is key to successful implementation, especially when they may have samples taken at satellite units where staff are less familiar with the new technique. De novo patients are unlikely to need extensive counseling; however, they should be provided with verbal and written information about C₂ monitoring and the procedure that will be adopted for sampling, after they have been discharged to the care of the outpatient clinic (see Implementation in the Outpatient Setting). The exception to this may be re-transplanted patients who were previously monitored by cyclosporine C₀ and who will require more extensive counseling at the outset on the difference between the two techniques.

Implementation in the Outpatient Setting. A new system will need to be set up for monitoring Neoral C₂ patients on an outpatient basis to facilitate their discharge from the ward setting.

The principles of educating staff, appointing a Neoral C₂ nurse, establishing a documentation and labeling system, and educating patients also can be applied to the outpatient setting. The main difference in the outpatient setting is that the nurse in charge will need to ensure that Neoral C₂ monitoring does not disrupt the efficient running of the clinic. This can be achieved by establishing a staggered appointment schedule with the phlebotomist to ensure patients are sampled on time and to avoid a back-log. Staggering the appointment schedule may have other benefits. As the experience of centers where Neoral C₂ monitoring has been implemented shows, the efficiency of the clinic can be improved, and patients are very positive about having a specific appointment for their blood work. Additionally, the nurse in charge will need to inform patients about when to take their dose of Neoral. Most patients can be relied on to take their

Neoral dose at home (and to note the time) prior to coming to their clinic appointment, so that the length of the clinic visit is not prolonged.

Once Neoral C₂ monitoring is established as a routine procedure in de novo patients and the outpatient clinic system is running, the unit may decide to transfer maintenance Neoral patients. Because these patients are already established on cyclosporine C₀ monitoring, a procedure will need to be put in place for recall and counseling. In order to give the patient time to digest the information—and to save clinic time—transplant units that have transferred maintenance patients from conventional trough to C₂ monitoring have used a recall system whereby patients are sent a letter before their next clinic visit that explains the benefits of the new procedure. The letter can be followed up with a telephone call to provide a more in-depth explanation of the benefits of Neoral C₂ monitoring, to ensure that the patient understands the arrangements for taking their Neoral dose, and to confirm the time when they are due at the clinic for their C₂ sample to be taken. From the outset, it is also important to decide how many patients the transplant unit can transfer at each outpatient clinic and to give these patients appointments at the start of the clinic session, to minimize disruption for both staff and patients.

Set up of Clinical Laboratory Procedures. Laboratory staff need to understand the rationale and benefits of Neoral C₂ monitoring, especially because they are remote from the patient setting. An understanding of their contribution to improving transplant patient outcomes, is critical to the success of the whole implementation program as they play a pivotal role in ensuring accurate testing and reporting of Neoral C₂ levels.

In the laboratory, the critical difference between cyclosporine C₀ monitoring and C₂ monitoring is the extra step involved in the dilution of the Neoral C₂ sample. (This may not be necessary if the laboratory is using the CEDIA Plus [Microgenics, Fremont, CA], which can directly measure 0.0–2.0 μ g/mL). Dilution of the Neoral C₂ sample is necessary because it contains much higher concentrations of cyclosporine than a C₀ sample. A dilution protocol needs to be established and validated in the laboratory before patient Neoral C₂ samples can be handled on a routine basis. Apart from a simple dilution procedure prior to using the established measurement procedure for cyclosporine, no other assay changes are required (14).

Additionally, a separate system for logging and reporting Neoral C₂ samples should be established. Owing to the large number of non-transplant indications for cyclosporine (e.g. psoriasis, nephrotic syndrome, uveitis, rheumatoid arthritis) it is likely that cyclosporine C₀ samples will continue to be sent to the laboratory from other departments, even if all transplant patients are eventually transferred to Neoral C₂ monitoring. Therefore, it may be worth considering the use of different units to report C₀ and C₂ levels back to the transplant team to ensure that the medical staff, who are using the levels to adjust the dose, are basing their decisions on correct data.

If patients have their Neoral C₂ blood sample taken at a satellite unit, the unit's laboratory will need to be informed of the change to C₂ monitoring as part of the implementation process and be supplied with Neoral C₂ sample labels to be used on their requisition forms.

Step 5: Piloting Neoral C₂ Monitoring

Piloting Neoral C₂ monitoring in a small group of patients (e.g., de novo patients) allows sampling and reporting procedures to be reviewed and altered, if necessary, and the unit protocol to be validated. It also enables the transplant team to become familiar with the Neoral C₂ target levels (see Tables 1 and 2) and dose adjustments according to C₂ level (see Fig. 1).

When piloting Neoral C₂ monitoring in clinical practice, it should be noted that a small proportion of patients can have markedly delayed absorption of cyclosporine (i.e., extended time to peak cyclosporine concentration [C_{max}]). These patients are referred to as "slow" absorbers. Increasing the Neoral dose according to the C₂ level in these patients may lead to excessive cyclosporine exposure. Measurement of C₂ alone cannot determine whether a low absorber of cyclosporine is a "true low absorber" or a "slow absorber" (a patient can be defined as a low absorber in the early post-transplant phase, if the C₂ level (in $\mu\text{g}/\text{mL}$) divided by the preceding dose of Neoral (in mg/kg) is less than 0.2 $\mu\text{g}/\text{mL}/\text{mg}/\text{kg}$ (P. Keown, personal communication).

If the C₂ value recorded on Day 3 or later indicates that the patient is a low absorber, carrying out further sampling at a later time-point (e.g., C₆) may help to determine if they are a true low absorber or a slow absorber. In true low absorbers, both C₂ and C₆ would be low and Neoral dosing should be adjusted according to C₂ value. In slow absorbers, C₆ is likely to be higher than C₂ and caution should be exercised when increasing the dose of Neoral, in order to avoid toxicity. It is also advisable to repeat the C₆ level after 2 to 3 weeks to confirm the maintenance status of the patient, owing to the rapid changes in the pharmacokinetic characteristics of cyclosporine in the early posttransplant period. In the maintenance renal transplant recipient, a C₂ level higher than 1.0 $\mu\text{g}/\text{mL}$, for example, could be considered excessive exposure to cyclosporine; however, this needs to be confirmed in long-term prospective studies.

Step 6: Implementation

Once the pilot phase has been evaluated and systems have been adjusted, full-scale implementation can take place. Routine auditing of Neoral C₂ monitoring, however, is necessary over time to detect problems at an early stage and to evaluate the impact of higher numbers of patients on the efficiency of sampling and reporting. These audits could also be used to decide when to implement Neoral C₂ monitoring in the maintenance patient population.

CONCLUSIONS

Neoral C₂ monitoring provides the optimal strategy for individualizing therapy for de novo and maintenance transplant patients (1-15). The establishment of target C₂ levels for renal and liver transplantation (12,13) and the development of a simple dose-adjustment formula (13) have stimu-

lated the adoption of Neoral C₂ monitoring in routine clinical practice. Experience from centers that have successfully adopted Neoral C₂ monitoring suggests that a step-wise approach is the key to the smooth implementation of this valuable patient management tool. By following the six-step approach, units contemplating the use of Neoral C₂ monitoring will be able to maximize both the unit's support for the transition from trough monitoring and the efficiency of sampling and reporting systems. The step-wise approach will ensure that patients can be sampled on time and managed according to the resulting C₂ level, with minimal disruption to ward, clinic, and laboratory routines.

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Telephone Number 308-8362

Application Number 09/804,584

1. Transplantation:
2003 Jan 15, 75(1):137-145
2002 Jan 15, 73 (1 suppl):S19-S22
2. Blood, 2003 Feb 15, 101(4):1439-1445
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Immunosuppressive effects of apoptosis; T cell anergy induced by apoptotic cells or apoptotic bodies

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ABSTRACT

There are many reports in respect to that T cell anergy is induced under certain circumstances. Now we discovered a novel manner of T cell anergy induction that apoptotic bodies disturb an appropriate interaction of T cell and antigen-presenting cell, whereby T cell receives insufficient signals via T cell antigen receptor, leading to anergy. Because apoptosis of neutrophils, T cells, and antigen-presenting cells is considered to occur frequently in an inflammatory site where cell-cell interactions vigorously occur, our proposed inducing manner of T cell anergy plays a pivotal role in physiological preventing ever

developing inflammation and too much damage to a host. This physiological induction of T cell anergy (naturally acquired ability) should be aggressively applied to clinical treatment for autoimmune diseases.

1. INDUCING MANNERS OF T CELL ANERGY

Anergy is a cellular state in which a cell can not normally respond to an optimal stimulation enough to activate a not anergy-induced (naive) cell. Several reports have described that T cell anergy is induced under certain circumstances.

1) When stimulated in the absence of B7, which is a costimulatory molecule expressed on APCs, T cells undergo anergy (1-3). B7 binds CD28, which is a counter receptor expressed on T cell and delivers a costimulatory signal to

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downstream molecules involved in intracellular signal transduction of T cell (4). One important downstream molecule of CD28 in intracellular signal transduction is JNK, one of MAPKs, which augments transcriptional activity of AP-1 complex (5). AP-1 is considered a major IL-2 gene transcription factor (6).

2) Analogues of antigenic peptides presented by APCs induce anergy to T cells (7-9). Analogue or altered peptide ligand is usually constructed by substituting an amino acid of an antigenic peptide for another amino acid. Each analogue subsequently has either characteristic of agonist, partial agonist, or antagonist. A part of partial agonists has a capacity of inducing anergy to T cells. Analogue is considered to have the characteristic by altering avidity between T-cell antigen receptor (TcR) on T cell, and epitope (analogue) in association with major histocompatibility complex (MHC) on APC.

3) Non- or weakly mitogenic antibody to CD3 induces anergy to T cells (10,11).

Smith *et al.* (11) investigated TcR ζ chain tyrosine phosphorylation and ZAP70 association, and showed that biochemical signals delivered by

nonmitogenic anti-CD3 antibody resemble altered peptide ligand signaling.

4) Anergy is induced to T cells occupied for TcR in the presence of interleukin-10 (IL-10) (12,13). IL-10 is produced by a number of different of cells including T cells and APCs (14). IL-10 suppresses T cell proliferation by specific inhibition of IL-2 production (15), and has potent inhibitory effects on alloantigen-induced T cell response (16). Therefore IL-10 is called "anti-inflammatory cytokine". IL-10 down-regulates MHC class II expression of monocytes and diminishes their antigen-presenting capacity (17), and inhibits upregulation of CD86 (B7.2) in dendritic cells (DCs) (13), so that IL-10 contributes to induction of T cell anergy.

5) High concentrations of soluble antigenic peptides induce anergy to human T cells (18,19). Lake *et al.* (19) observed that high concentration of soluble peptide causes profound down-regulation of CD28.

6) T cell anergy is induced by activated MHC class II+ T cells which present antigenic peptides to other T cells (T-T interaction) (20-24). Activated T cells express sufficient density of MHC class II molecules to deliver first signal to other

T cells, but to deliver

These may not be rather no induction

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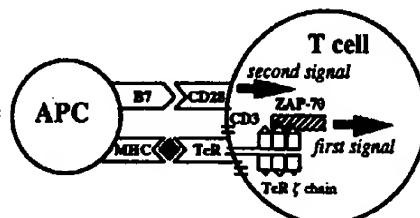
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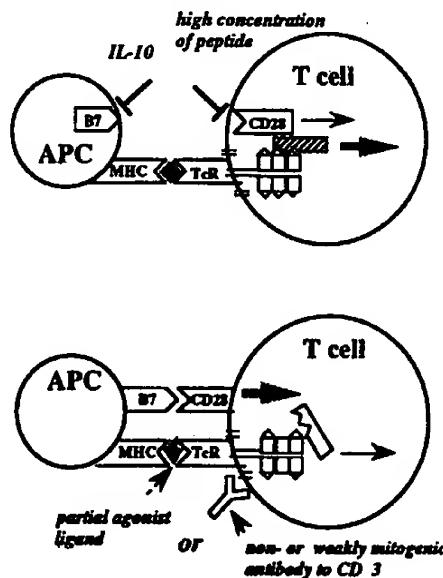
T cells, but express B7 at too low density to deliver second signal.

These manners of anergy induction can not be independent of each other, or rather may be complicatedly combined (Fig. 1). For examples; i) Some anergy inductions are summarized by lack of

A. Proliferation induction



B. Anergy induction



sufficient interaction of B7 and CD28. IL-10 inhibits up-regulation of CD86 in DCs. High concentration of antigenic peptide causes downregulation of CD28 on T cell. Activated T cell expressing MHC class II molecules does not have sufficient density of B7. ii) The other anergy inductions are summarized by lack of sufficient signals via TcR/CD3 complex. Inducing manner of T cell anergy by analogues of antigenic peptides can be similar to that by non- or weakly mitogenic anti-CD3 antibodies. In these

Fig. 1. Inducing manners of T cell proliferation and anergy, and role of TcR ζ chain in determining T cell destiny. (A). T cell is fully stimulated by receiving sufficient amount of both first and second signals. TAMs in TcR ζ are tyrosine-phosphorylated and then TcR ζ recruit ZAP70 via the phosphorylated TAMs (Proliferation induction). (B). Blockade of interaction of B7 (by IL-10) and CD28 (by high concentration of soluble antigenic peptide) induces weak second signals, leading to T cell anergy. Partial agonist ligand, and non- or weakly mitogenic antibody to CD3 are similar for altered tyrosine-phosphorylation of TcR ζ , not delivering first signals by insufficient recruitment of ZAP70 (Anergy induction). Triangle of TcR ζ chain represents tyrosine-phosphorylated TAM. The width of arrows indicates the strength of signals.

cases, T cells do not receive sufficient signals to induce TcR ζ tyrosine phosphorylation (see below).

2. ALTERED INTRACELLULAR SIGNAL TRANSDUCTION AT INDUCING ANERGY TO T CELLS

Many investigators have reported altered intracellular signal transduction in anergic T cells and at inducing anergy to T cells (reviewed in 25). Many species of intracellular signal transducing molecules were discovered. Although their phosphorylations and associations were vigorously studied, common molecular mechanism at anergy induction of T cell has not yet been elucidated. Recently TcR ζ chain has been noticed again as a decisive molecule for T cell destiny. Sloan-Lancaster *et al.* (26) initially described that analogues of an antigenic peptide, capable of inducing T-cell anergy, induce altered phosphorylation of TcR ζ . The analogues induced much less pp21 (antigen-induced tyrosine-phosphorylated form of TcR ζ) as compared with the antigenic peptide, leading to an decrease of the ratio of pp21/pp18 (pp18; constitutively tyro-

sine-phosphorylated form of TcR ζ). The altered phosphorylated-TcR ζ chains can not normally recruit ZAP70 via their tyrosine-based activation motifs (TAMs), probably leading to unresponsive status of T cells. TcR ζ chains usually exist as a homodimer and each TcR ζ chain contains three TAMs. Once TAMs are phosphorylated, they are effectively bound by two SH2 domains of ZAP70. The six TAMs are not phosphorylated at the same time, but are phosphorylated at serial order depending on the nature of TcR ligand (27). This fashion of phosphorylation could critically modulate delivering signals to downstream of intracellular signal transduction because tandem SH2 domains are strictly required for binding TAMs (28). Single SH2 domain has much lower affinity with a corresponding TAM. This may be supported by the facts that ZAP70 is not associated with TcR ζ and is not phosphorylated when anergized T cells are restimulated with antigenic stimuli (29-31). The serial phosphorylations of TAMs in TcR ζ therefore can play a pivotal role in determining responses of T cells, proliferation, anergy, apoptosis, and no response. Further investigations of this system using T cells stimulated in

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different anergy inducing manners are expected.

3. APOPTOTIC CELLS ALSO HAVE A POTENTIAL FOR INDUCTION OF T CELL ANERGY

Roles of apoptosis in immunosuppressive effects including induction of T cell anergy have been recently reported. Voll et al. reported that macrophage secrets more amount of interleukin-10 (IL-10) but less amount of proinflammatory cytokines tumour necrosis factor- α , IL-1 and IL-12, when macrophage is stimulated with bacterial lipopolysaccharides (LPS) in the presence of apoptotic peripheral blood lymphocytes compared with in the absence of them (32). The authors proposed that influence of apoptotic cells upon thrombospondin receptor (CD36) causes such altered profile of cytokines secretion. On the contrary, Fadok et al. (33) described that when macrophages phagocytose apoptotic cells irrespective of the presence of LPS, transforming growth factor- β 1 secreted from the macrophages is increased and inhibits not only proinflammatory cytokines but also

IL-10. They used human neutrophils or Jurkat cells irradiated with UV as apoptotic cells.

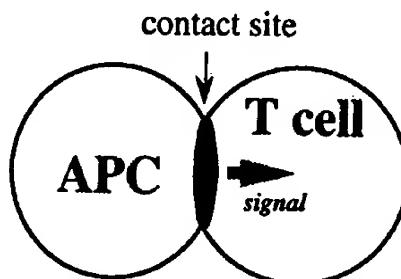
The discrepancy regarding IL-10 secretion between the two reports remains to be elucidated. The altered cytokine profile caused by apoptotic cells is not exactly understood yet for the mechanism(s). It can, however, be stated that apoptotic cells have a potential to inhibit secretion of proinflammatory cytokines but enhance anti-inflammatory cytokines, leading to immunosuppressive status.

These reports showed an effect of apoptotic cells upon cytokine secretions in macrophage stimulated with LPS which directly binds a receptor CD14 on macrophage and stimulates macrophage. Meanwhile we investigated effects of apoptotic bodies (particles fragmented from apoptotic cells) upon interaction between APCs and T cells by addition of *Staphylococcus aureus* enterotoxin B (SEB), mainly by measuring T cell proliferation (34). First, T cells are stimulated with SEB and then treated with mitomycin C, resulting in apoptosis. When T cells are stimulated with SEB in

the presence of apoptotic bodies derived from the SEB-stimulated autologous T cells, T cell proliferation was inhibited and T cell unresponsiveness was also induced. Based on less downregulation of TcR on T cell stimulated in the presence of apoptotic bodies compared with that on T cells stimulated in the absence of them, we concluded that inhibition of proliferation and induction of unresponsiveness in T cells are caused by physical blockade of apoptotic bodies for interaction between T cells and APCs (Fig. 2).

Although T cell can attach to a part of cell surface not covered by apoptotic bodies on APC, this attachment is not considered to deliver sufficient signals to the T cell (Fig. 2B). Because, at contact between T cell and APC, receptors including TcR/CD3 complex and intracellular proteins clustered into spatially segregated three-dimensional domains within the cell contact site, and these cluster formations may generate appropriate physiological response of T cell (Fig. 2A) (35). Our observed novel phenomenon and its interpretation are important as to that apoptotic bodies cause induction of anergy in T cells, and

A. Normal interaction between T cell and APC



B. Interaction disturbed by apoptotic bodies between T cell and APC

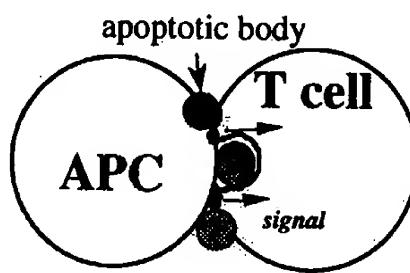


Fig. 2. T cell anergy induced by blockade of apoptotic bodies in interaction between T cell and APC. (A) At contact between T cell and APC, receptors of T cell cluster into spatially segregated three-dimensional domains within the cell contact. This cluster formation may generate appropriate physiological response of T cell. (B) Blockade by apoptotic bodies of interaction between T cell and APC disturbs the cell-cell contact, resulting in insufficient or little amount of cluster formation, so that T cell can not receive appropriate signals to be activated and undergo anergy.

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Immunosuppressive effects of apoptosis

the same situation can be often found *in vivo* especially in an inflammatory site.

More apoptosis is induced in an inflammatory site into which inflammatory cells such as neutrophils, APCs and T cells migrate (Fig. 3). Neutrophils express Fas and are sensitive to apoptosis induced by Fas ligation (36-38). Activated T cells upregulate Fas expression and are sensitive to apoptosis (39). These cells expressing Fas and sensitive to Fas ligand can undergo apoptosis by ligation of Fas ligand, which is expressed on activated T cells (40-42) and APCs (43), and of soluble Fas ligand which is released by macrophage taking up apoptotic neutrophils (37). Furthermore, macrophage that engulfs apoptotic cells are recognized via epitopes from engulfed apoptotic cells in association with MHC class I molecules and subsequently lysed by antigen-specific cytotoxic T cells (44). At a late phase of an inflammation involving cells such as neutrophils, APCs, and T cells, apoptotic cells consequently can accumulate and APCs may decrease in number in the inflammatory site. In such situation, survived APCs are considered to be bound via CD14 (45) or CD36 (46) by

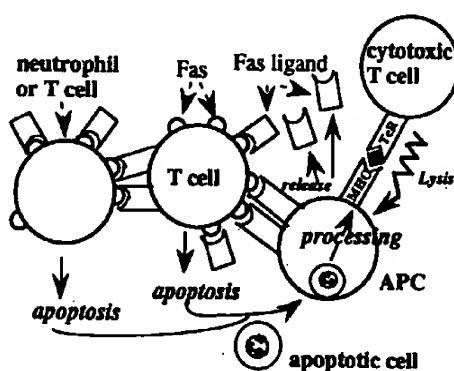


Fig. 3. Immunosuppression induced by apoptotic cells or apoptotic bodies. Besides the physical blockade of apoptotic bodies (see Figure 2), apoptotic bodies or apoptotic cells play immunosuppressive roles. Macrophage taking up apoptotic cells release soluble Fas ligand. Furthermore the macrophage that engulfed apoptotic cells presents epitopes in association with MHC class I molecules, and is subsequently lysed by antigen-specific cytotoxic T cell.

relatively more number of apoptotic cells and apoptotic bodies, and then to be physically blocked for delivering sufficient signals to T cells probably via not only MHC but also B7.

4. PROPOSAL FOR APPLICATION OF THE IMMUNOSUPPRESSIVE EFFECTS OF APOPTOSIS

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potential. APCs that engulf apoptotic cells are killed by cytotoxic T cells, secret anti-inflammatory cytokines but inhibit proinflammatory cytokines and release soluble Fas ligand capable of killing Fas-expressing cells. Apoptotic bodies physically block an interaction between T cells and APCs. The T cells that receive the insufficient signals acquire a predisposition for unresponsiveness. These roles of apoptosis might have been positively selected by nature because ever developing inflammation without the immunosuppression can lethally damage a host. Why do not we utilize this given mechanism of suppressing inflammatory cells for autoimmune diseases or treatment of graft rejection?

5. ACKNOWLEDGEMENTS

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Recent Res.

Regul inhibi

Alex B. Le
Department
Pathology, UABSTRACT
Since its discovery, the biological activities of secreted leukocyte proteinases have been studied. In this small review, we have found that SLPI, a secreted immunological inhibitor, has a potent inhibitory effect on the HIV infection of T cells. The mechanism of this regulation is discussed.

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Dendritic cells resurrect antigens from dead cells

Marie Larsson, Jean Francois Fonteneau and
Nina Bhardwaj

Antigens that do not normally access the cytoplasm of antigen-presenting cells, such as certain tumor and viral antigens, become targets of cytotoxic T lymphocytes (CTLs). Over the past 25 years, substantial evidence has emerged for an 'exogenous' pathway for loading MHC class I molecules.

Dendritic cells are potent stimulators of T-cell responses and can induce CD8⁺ CTLs by phagocytosis of dead tumor or virus-infected cells. Here, Marie Larsson and colleagues discuss the role of dendritic cells in stimulating MHC class I-restricted T-cell responses by exogenous routes.

CD8⁺ T cells are crucial components of the immune response to pathogens and tumors. Activation of CD8⁺ T cells into cytotoxic T lymphocytes (CTLs) requires two steps. First, presentation of antigenic peptides on professional antigen-presenting cells (pAPCs) such as dendritic cells (DCs). Second, helper function provided by CD4⁺ T cells. CTLs, via their T-cell receptors (TCRs), recognize MHC class I molecules bearing antigenic peptides of 8–10 amino

acids. Peptides that bind to MHC class I molecules are derived from endogenously synthesized proteins, either the cell's own proteins or proteins synthesized within the cell's cytoplasm by infectious agents. This is referred to as the 'endogenous' pathway of antigen presentation (Fig. 1a). In contrast to CD8⁺ T cells, CD4⁺ T cells recognize MHC class II molecules, which express antigenic peptides derived from the 'exogenous' pathway (Fig. 1b).

'In contrast to CD8⁺ T cells, CD4⁺ T cells recognize MHC class II molecules, which express antigenic peptides derived from the "exogenous" pathway.'

The paradigm that CD8⁺ T cells can recognize only endogenously synthesized antigens, whereas CD4⁺ T cells recognize exogenous antigens, failed to take into account the observation that immunity develops spontaneously to viruses that cannot infect pAPCs [e.g. Epstein–Barr virus (EBV)], or to tumors and allografts that by themselves are poor APCs. In 1976, Bevan showed that mice immunized with cells that express foreign minor histocompatibility antigens mounted an antigen-specific response that was restricted to self-class I, demonstrating that exogenous pathways are important in the induction of CD8⁺ T-cell responses¹. This phenomenon coined

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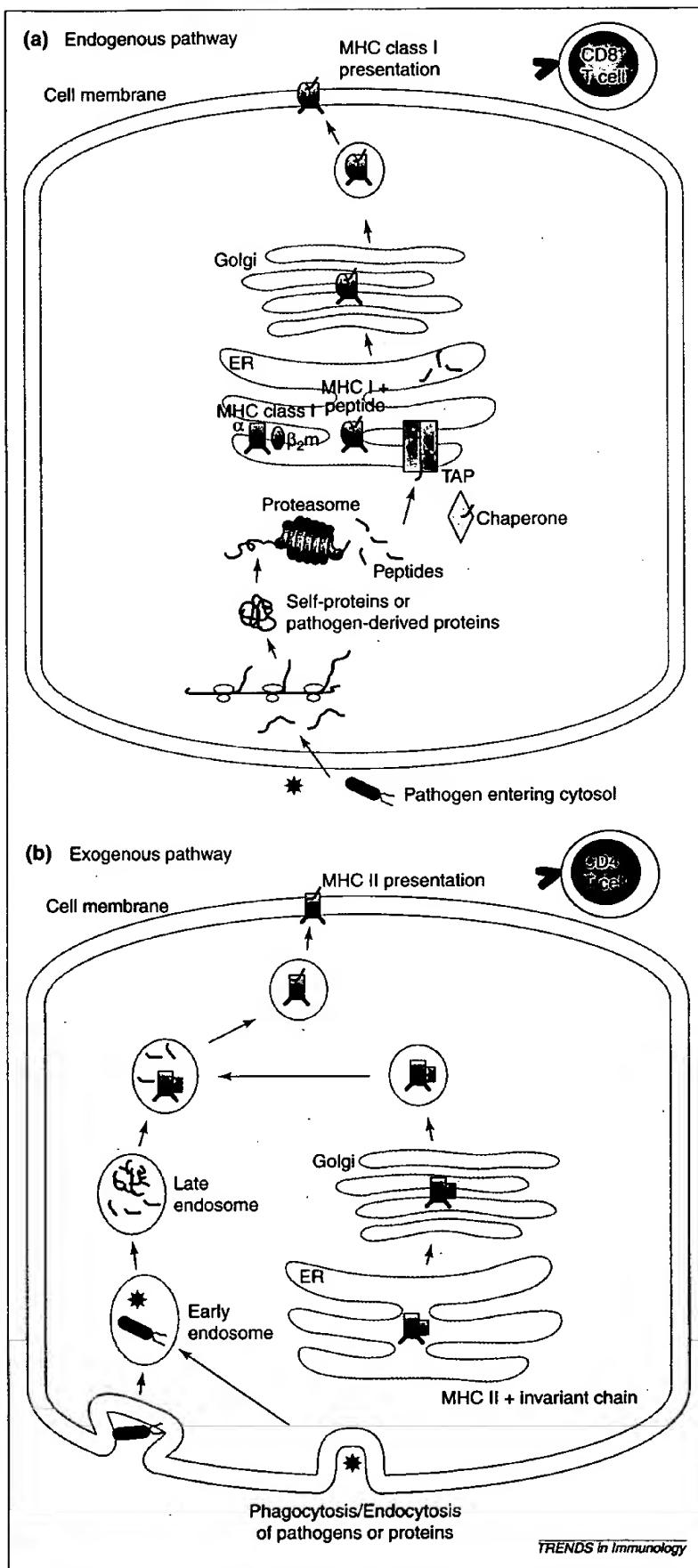


Fig. 1. (a) The endogenous pathway. Pathogen-derived or self-proteins within the cytosol of antigen-presenting cells (APCs) are enzymatically digested into peptides, mainly by cytosolic proteases called proteasomes, and are then transported by TAP molecules into the endoplasmic reticulum (ER). In the ER lumen, peptides bind to MHC class I molecules, which are subsequently transported via the Golgi to the plasma membrane. (b) The exogenous pathway. Exogenous antigens are derived from proteins that are endocytosed and processed by proteases. Peptides bind to newly synthesized MHC class II molecules in specialized antigen-processing vesicles (MHC class II enriched compartment; MIIC), and the complexes are externalized to the plasma membrane. Abbreviations: β₂m, β₂-microglobulin; TAP, transporter associated with antigen processing.

'cross-priming', formed the basis of the postulate that antigens could be exogenously transferred from donor cells to recipient pAPCs for presentation on MHC class I molecules. Subsequent studies confirmed that immunostimulatory CD8⁺ T-cell responses could be generated to antigens expressed in peripheral autologous tissues that lacked pAPC function, such as viral antigens, H-Y self-antigens and tumor antigens²⁻⁴. Peripheral tolerance to self-antigens was thought to involve a similar exogenous pathway of 'cross-tolerance'⁵. The term cross-presentation is used to describe the processing and presentation of exogenously derived antigen in the MHC class I pathway. This can result in either the stimulation of a CD8⁺ T-cell response or in tolerance.

Several important features emerged from these and other studies: (1) efficient cross-presentation of antigen required bone-marrow-derived cells as APCs (Refs 4,6-9); (2) diverse groups of antigens could access MHC class I by exogenous pathways, at least in *in vitro* models¹⁰; (3) in some cases, cross-presentation was dependent upon the presence of TAP (transporter associated with antigen processing)^{6-8,11}, suggesting that antigens have to access the cytoplasm of pAPCs to converge with conventional processing pathways; and (4) cross-presentation of antigens required CD4 help, which could be substituted by the delivery of CD40 ligand (CD40L) or the release of inflammatory cytokines in the milieu, the latter considered to 'activate' pAPCs (Refs 12-15). However, several questions remain with respect to the phenomenon of cross-priming *in vivo*: namely, the identity of the pAPC cross-presenting viral and tumor antigens to T cells, the physiological form of antigens accessing pAPCs and the processing pathways that permit access of antigens to MHC class I. Evidence that the pAPC in question is the DC and that dead cells or cell fragments and derivatives are an important source of antigens for cross-priming is discussed below.

APCs that mediate cross-presentation onto MHC class I molecules

DCs and macrophages are commonly grouped as pAPCs with similar functions. Cells of the macrophage line are important elements of innate

immune responses; they are professional 'scavengers', but poorly stimulate naive or resting memory T cells¹⁶. By contrast, DCs are uniquely specialized to initiate T-cell immunity *in vitro* and *in vivo*¹⁷. DCs acquire antigens in peripheral tissues in their immature state through a variety of mechanisms, including endocytosis, macropinocytosis and phagocytosis. DC 'maturation' is induced by exposure to microbial RNA or DNA, viruses, bacteria, lipopolysaccharide (LPS), heat shock proteins (HSPs), immune complexes or cytokines. Maturation is characterized by downregulation of antigen acquisition, increased expression of MHC and co-stimulatory molecules, interleukin 12 (IL-12) production, and altered expression of chemokine receptors¹⁸. As they mature, DCs migrate to the T-cell areas of lymphoid organs, where antigen is presented to naive CD4⁺ and CD8⁺ T cells. When delivered *in vivo*, DCs charged with peptide antigens, tumor lysates, eluted peptides or viral vectors that encode relevant antigens induce immune responses, which include protective and therapeutic immunity to tumors and pathogens in animals, and clinical antitumor responses in humans¹⁹.

DCs and cross-presentation

A pivotal role for DCs in cross-presentation has become apparent from studies in human and animal systems. Albert *et al.* demonstrated that human DCs could phagocytose apoptotic influenza-infected monocytes, and stimulate resting CD8⁺ T cells to develop into CTLs (Ref. 20). By contrast, monocytes or macrophages, although more efficient at phagocytosis, could not elicit CD8⁺ CTLs (Ref. 20). The failure of monocytes was attributed to their inability to generate the threshold number of peptide-MHC class I complexes to activate specific T cells and low levels of co-stimulatory molecules. In another model, *Salmonella typhimurium* infection was shown to induce apoptosis in murine macrophages under certain conditions. Bystander DCs and macrophages phagocytosed these cells efficiently but only DCs presented bacteria-derived antigens on MHC class I molecules²¹. Similar distinctions between these APCs have been noted with respect to presentation of exogenously delivered proteins such as ovalbumin (OVA)²² or immune complexes²³. Furthermore, in *in vivo* studies, only DCs pulsed with apoptotic tumor cells, but not monocytes, primed tumor-specific CTLs (Ref. 24).

More direct evidence that DCs can mediate cross-presentation *in vivo* comes from two observations. First, injection of short-lived migratory murine DCs led to their processing by recipient DCs in the lymph node and *in situ* expression of antigens²⁵. Second, splenic DCs (which express high levels of CD11c) isolated after

in vivo priming with OVA-loaded β_2 -microglobulin-deficient splenocytes presented OVA antigens to OVA-restricted MHC class I CD8⁺ T cells. Cross-priming was TAP dependent and restricted to the lymphoid CD8⁺ DC subset, even though myeloid CD8⁻ DCs acquired antigens *in vivo*²⁶. Macrophages, which do not express CD11c, did not appear to play a significant role in cross-presentation of protein antigens²⁶.

Macrophages and cross-presentation

In contrast to these findings, there is a body of literature indicating that monocytes or macrophages (and even B cells) cross-present many different forms of antigens to CD8⁺ T cells *in vitro*²⁷⁻³¹. For example, murine macrophages that had phagocytosed apoptotic transformed cells were recognized as targets of antigen-specific CD8⁺ T-cell clones²⁷. These results are difficult to reconcile but they might depend upon the activation status of APCs, the sources of antigens used to study cross-presentation and the T-cell readout. In any case, the contribution of monocytes and macrophages should not be underestimated in cross-presentation to self-antigens. Cells of the macrophage line scavenge apoptotic cells and suppress inflammatory responses by producing transforming growth factor β (TGF- β), IL-10 and IL-13 [in the place of pro-inflammatory cytokines such as tumor necrosis factor α (TNF- α), IL-1 and IL-12 (Ref. 32)] and decreasing nitric oxide synthase³³. Therefore, apoptotic cell-macrophage interactions have the potential of profoundly suppressing the immune response, including responses induced by DCs. The use of discrete sets of receptors, intracellular signaling pathways by monocytes and macrophages (e.g. $\alpha_5\beta_3$ integrins and phosphatidyl serine receptors), and the consequences of exposure to apoptotic cells (e.g. IL-10 production) might result in these differences.

Sources of antigens for cross-presentation

In vitro, several forms of antigen can access the exogenous pathway for class I presentation, apparently through phagocytic or nonphagocytic mechanisms (reviewed in Ref. 10), including: (1) cells and cellular components, such as apoptotic cells or bodies²⁰, cell lysates³⁴, HSPs (Ref. 35) and exosomes³⁶; (2) organisms, such as bacteria²¹, viruses or virus-like antigens, and intracellular pathogens^{1,6,7,33,37}; (3) proteins, whether soluble, particulate or complexed with immunoglobulin^{28,30,37}; and (4) antigens encoded by RNA or DNA (Ref. 10). Because cell death is a consequence of normal development and tissue turnover, viral and bacterial infections, inflammation, hypoxia or injury, it is possible that dead cells or their 'debris' are one of the primary sources of exogenous antigens *in vivo*.

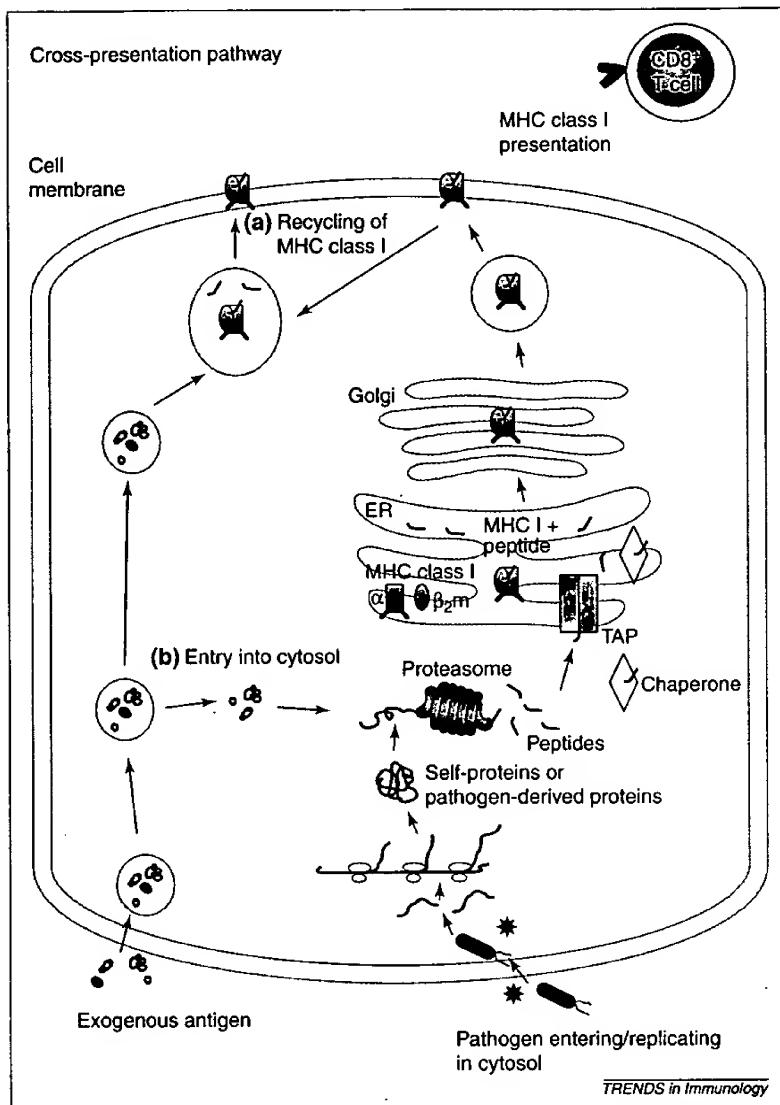


Fig. 2. Cross-presentation pathways. (a) Exogenous antigens can access MHC class I molecules within endosomes that contain MHC class I molecules that have been recycled from the membrane. In addition, antigen can be regurgitated to cell surface MHC class I molecules. (b) Alternatively, antigen is transported out from the endosome to the cytosol and processed similarly to endogenously derived antigens. Abbreviations: β_2 m, β_2 -microglobulin; ER, endoplasmic reticulum; TAP, transporter associated with antigen processing.

Cell death is commonly divided into two forms based on morphological criteria: apoptosis, a programmed cell death that occurs during tissue homeostasis; and necrosis, a physical disruption of cells thought to occur in pathological situations. Cells undergoing apoptosis modify their surface by exposing phosphatidylserine (PS) and altered carbohydrates³², and are recognized while the membrane is still intact via specific receptors on phagocytes. In necrosis, inflammation ensues due to the release of cellular contents and activation of bystander cells.

Albert *et al.* first demonstrated *in vitro* that antigen-specific CD8⁺ CTLs could be induced by DCs that had phagocytosed influenza-infected cells that were spontaneously undergoing apoptosis²⁰. The

source of dying cells could be allogeneic or xenogeneic. Optimal CTL activation required phagocytosis of apoptotic cells by immature DCs, followed by a maturation signal to enhance costimulatory activity. The maturation stimulus enhanced cross-presentation up to 100-fold and could be delivered by CD40L, LPS or a cocktail of inflammatory cytokines (M. Larsson, unpublished). In addition, bacteria-infected apoptotic cells have been identified as sources of antigen for cross-presentation by DCs (Ref. 21).

In vivo, DCs can acquire cell components in rat mesenteric lymph and lymph nodes. A subset of DCs that contain cytoplasmic apoptotic DNA, epithelial cell-restricted cytokeratins and nonspecific esterase (NSE) has been identified³⁸. Therefore, these DCs transport apoptotic interstitial epithelial cells to T-cell areas of mesenteric lymph nodes, probably to maintain peripheral self-tolerance, and/or to stimulate immune responses following encounter with dying pathogen-infected epithelial cells. Moreover, studies have shown that DCs cross-present antigens from necrotic cells to CD8⁺ T cells^{34,39,40}.

Dead cells, or their components, have distinct effects on DCs. Some studies have shown that co-culture with apoptotic cells does not induce DC maturation^{41,42}, whereas others have reported that maturation of DCs ensues following exposure to apoptotic cells, including virus-infected cells, possibly through TNF- α -dependent pathways⁴³⁻⁴⁵. These differences are unaccounted for but might depend upon the condition of the apoptotic cells (cytokine release due to virus infection or secondary necrosis) and/or the source of DC (murine, monkey versus human). By contrast, exposure of DCs to necrotic tumor-cell lines or tissues results in DC maturation^{41,42}. As discussed below, these findings have significant implications for how tolerogenic versus immunogenic responses are elicited. The nature of the antigenic material in apoptotic cells or necrotic cell lysates that mediates cross-presentation by DCs remains to be determined but HSPs are principal candidates. HSPs are highly conserved chaperones for the transport and folding of proteins, including the chaperoning of antigenic peptides to MHC class I complexes in the endoplasmic reticulum (ER). HSPs derived from virus-infected cells or tumor cells (cytoplasmic HSP70 and 90, and ER gp96) induce protective immunity and CTLs *in vivo*³⁵. HSPs bind saturably to receptors on monocytes and DCs (e.g. CD91)⁴⁶ and induce their activation or maturation⁴⁷. HSPs are confined within apoptotic cells, bodies and blebs but are released when cells are disrupted or undergo secondary necrosis⁴⁸. These observations are consistent with the hypothesis that HSPs released from necrotic or stressed cells provide a stimulatory and specific signal to the immune system³⁵.

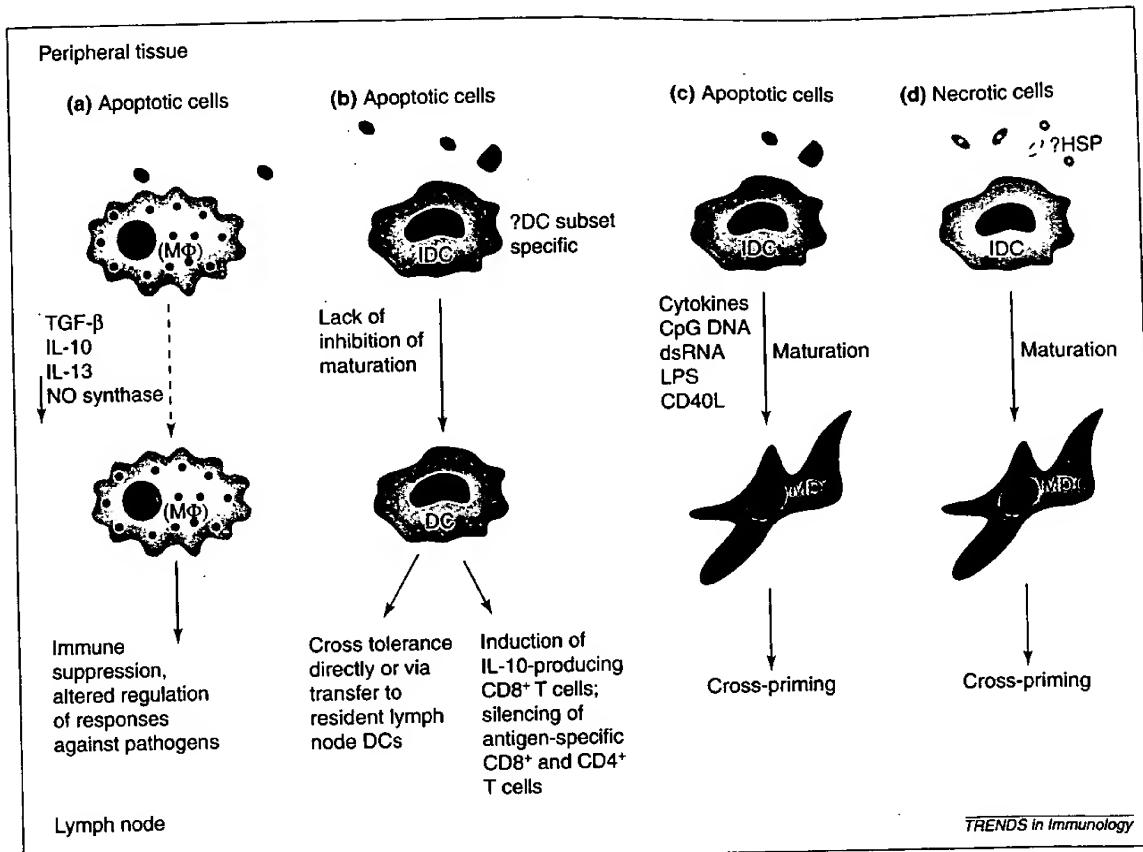


Fig. 3. Dendritic cells (DCs) mediate tolerance to self-antigens and immunity to pathogens. (a) Macrophages (MΦ) phagocytose and clear antigens from apoptotic cells but become primed to release immunosuppressive cytokines that can compromise effector immune function and pathogen clearance. (b) Immature DCs (IDCs) and/or specific DC subsets in peripheral tissues phagocytose apoptotic cells. In the absence of a maturation signal, the DCs induce tolerance, either directly or through acquisition by resident lymph node DCs. Although the nature of the tolerizing signal has not been identified, induction of IL-10-producing CD8⁺ T cells, and/or silencing of antigen-specific CD8⁺ and CD4⁺ T cells either directly or via immunoregulatory cells might be mechanisms. (c) When DCs are exposed to a maturation signal, CD8⁺ T cell activation and cross-priming ensues. (d) Under certain circumstances, necrotic cells can provide both a source of antigen and a simultaneous maturation signal, and thereby induce DCs to cross-present antigens to CD8⁺ T cells. Abbreviations: CD40L, CD40 ligand; dsRNA, double-stranded RNA; HSP, heat shock protein; IL-10, interleukin 10; LPS, lipopolysaccharide; NO, nitric oxide; TGF-β, transforming growth factor β.

Current models of cross-presentation in DCs

The issue of how and where apoptotic or necrotic cells get processed in DCs remains unknown. Following phagocytosis, apoptotic cells localize in vesicles that express MHC class I and class II molecules and lysosomal markers, and disappear after 6–8 h without evidence of cytoplasmic localization. Antigens contained within apoptotic cells have not yet been localized in the cytoplasm. DCs use at least one set of receptors, $\alpha_v\beta_5$ and CD36 to phagocytose apoptotic cells⁴⁹. The $\alpha_v\beta_5$ integrin might direct the cytoskeletal changes necessary for the phagocyte surface to envelop apoptotic cells through recruitment of the CrkII–DOCK180–Rac1 signaling complex⁵⁰ and possibly route dead cells to

specialized processing pathways. Antigens within necrotic lysates probably access endosomes through both receptor-specific and nonspecific pathways.

In vivo, the TAP dependency of cross-priming in animal models to tumor antigens, and certain organisms such as vaccinia and poliovirus^{7,11}, suggests that exogenous antigens can enter the cytoplasm from endosomes following internalization (Fig. 2). *In vitro*, soluble antigens or antigens within immune complexes appear to access the cytoplasm of murine DC lines²³. Only molecules of 3–20 kDa traverse the endosomal membrane, suggesting the existence of active transporters, pores or leaky junctions for cytoplasmic access. This pathway is inhibited by chloroquine, blocked by lactacystin, a proteasome-specific blocker, and is unique to DCs because macrophages fail to present the antigen²³. Further, antigens might be processed endosomally for access to recycling MHC class I molecules on the same cell or neighboring cells after 'regurgitation'¹⁰. Peptides complexed to HSPs in certain orientations might be processed in this fashion. Recently, a TAP-independent but bone-marrow-derived pAPC-dependent pathway of antigen processing for certain influenza and lymphocytic choriomeningitis virus (LCMV) responses have been described *in vivo*^{6,7}, supporting the idea that peptides can bind to MHC class I molecules in locations other than the ER. The contribution of these pathways in the cross-presentation of antigens from dead cells by DCs,

might depend upon the DC subset³⁸, species, antigen source^{6,8,23} and might not be mutually exclusive^{6,8}.

Cross-priming in tolerance, autoimmunity, viral and tumor immunity: a role for DCs

Tolerance and autoimmunity

Tolerance to self-antigens is achieved centrally in the thymus, and peripherally for self-antigens that are not encountered within thymic tissues. Evidence that cross-presentation is essential to achieve tolerance came from bone-marrow-derived chimeras and transgenic animals. Central deletion of CD8⁺ T cells was shown to depend upon the cross-presentation of thymic stroma by bone-marrow-derived APCs (Ref. 51), and a role for thymic DCs in negative selection has been established¹⁸. Peripheral deletion of CD8⁺ T cells (in this case naive OVA-specific T cells) was dependent upon bone-marrow-derived APCs that acquired antigens from organs expressing OVA and that trafficked to draining lymph nodes⁹. Similar results concluding that cross-presentation is essential for tolerance have been obtained in other systems⁵². What factors break tolerance and induce autoimmunity? Persistent high antigen loads, such as a failure to clear apoptotic cells, might be one mechanism. This idea is supported by experiments in mice where the induction of apoptosis or direct injection of large amounts of apoptotic cells induces autoimmune responses⁵³. Animals with defects in receptors that mediate apoptotic cell clearance (e.g. C1q) develop a lupus-like disorder⁵⁴. Because apoptotic cells undergoing secondary necrosis might induce the activation of APCs such as DCs, the failure to remove these cells might set the stage for autoimmunity.

Immunity to pathogens

It is unlikely that pAPCs are infected by all viruses, therefore alternative mechanisms must exist for presentation of viral antigens to CD8⁺ T cells. Especially when one considers that many viruses have evolved tactics to evade presentation on MHC class I molecules. Experimental models have established that CD8-mediated immunity to viruses can develop through exogenous routes. Sigal *et al.* constructed bone marrow chimeras expressing human polio virus receptors (PVR) on non-hematopoietic tissues only. Infection with polio virus elicited antiviral responses when the animals were reconstituted with PVR-negative normal, but not TAP-knockout, bone marrow⁷. Therefore, viral antigens from peripheral tissues were acquired by bone-marrow-derived APCs, processed via a TAP-dependent route and presented to CD8⁺ T cells. Virus infected dying cells might have provided a source of exogenous antigens.

In vitro studies using vaccinia virus support these conclusions. Vaccinia is highly immunogenic, yet at

the same time induces apoptosis of DCs and inhibits their maturation. Using a recombinant vaccinia virus encoding the influenza matrix protein as a model, DCs were found to cross-present influenza antigens from both apoptotic and necrotic vaccinia infected cells to CD8⁺ T cells (M. Larsson *et al.*, unpublished). Importantly, the DCs were not infected themselves. Several viruses induce apoptosis, including influenza, EBV, measles viruses and HIV-1. Apoptotic or necrotic EBV-transformed B-cell lines can be cross-presented by human DCs to both CD4⁺ and CD8⁺ T cells^{34,55}, as can HIV-infected cells induced to undergo apoptosis or necrosis (M. Larsson *et al.*, unpublished). Therefore cross-priming via exogenous pathways mediated by DCs are likely to be critical for generating optimal antiviral immunity *in vivo*.

Tumor immunity

Cross-priming might be essential for the generation of tumor immunity *in vivo*. Huang *et al.*⁴ showed that immunity to tumors that express influenza virus nucleoprotein required the cross-presentation of antigens from tumor cells to bone-marrow-derived APCs with functional TAP molecules. In humans, evidence has come from patients with autoimmune paraneoplastic cerebellar degeneration (PCD), who have limited underlying cancer (usually breast or ovarian) and antibodies towards CDR2 antigen, which is normally expressed in immune-privileged sites (neurons and testis). Autoimmunity is thought to develop as a consequence of an effective immune response towards the tumor that ectopically expresses CDR2. DCs from HLA-A*0201⁺ PCD patients phagocytosed apoptotic tumor lines that expressed CDR2 and induced potent anti-CDR2 cytotoxicity from autologous T cells⁵⁶. These data suggest that, in PCD, cross-presentation of tumor antigens by DCs provides the initial stimulus for CTLs *in vivo*. Several additional studies have confirmed that human DCs can cross-present antigens from apoptotic tumor cells (e.g. melanoma cells, squamous cell carcinoma^{39,57,58}) to CD8⁺ T cells.

Cell death and DCs as control points for cross-priming

How does the immune system decide when a tolerogenic versus an immunogenic response occurs to cross-presented antigens? We propose that the outcome is governed by the form of cell death, the APC, and the context in which the two meet. During normal cell turnover, apoptotic cells are scavenged by macrophages, which become primed to suppress immune responses (Fig. 3a). Immature DCs, which acquire apoptotic cells peripherally, would maintain tolerance in draining lymph nodes (Fig. 3b). Tolerance to T cells might be direct or indirect, involving the transfer of migrating DCs to resident lymph node DCs (Ref. 25) or activation of immunoregulatory cells. It might also depend

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upon specialized DC subsets^{26,59}. The concept that immature DCs are tolerogenic is supported by the observations that they can silence antigen-specific T cells *in vitro*⁶⁰. *In vivo*, human immature DCs, in contrast to mature DCs, silence influenza virus-specific CD8⁺ T-cell effector function and induce the development of influenza-specific IL-10 producing cells⁶¹. Concomitant exposure of immature DCs to a maturation stimulus at the time they phagocytose apoptotic cells could potentially turn a tolerogenic signal into an immunogenic one^{41,42}. What would be the relevant maturation signals *in vivo*? Apoptosis is often characterized as a non-inflammatory death. This point of view is being challenged with the recognition that apoptosis in the setting of virus or bacterial infection might promote inflammatory responses through release of cytokines such as TNF- α , IL-1 β and IL-18 (reviewed in Ref. 62). These cytokines have multiple effects, including DC maturation and induction of TNF- α . Therefore, under certain circumstances, apoptotic death might promote DC maturation (Fig. 3c). Other maturation signals *in vivo* include the provision of CD4⁺ help^{13,63}, which would mature DCs through CD40 ligation, double-stranded RNA from viruses, CpG DNA from microbes, LPS from bacteria, and HSPs. Finally, necrotic cells would simultaneously provide a source of antigens and a maturation signal

(Fig. 3d). Mature human monocyte-derived DCs and murine lymphoid CD8⁺ DCs induce Th1 responses owing to their production of IL-12 and interferon γ (Ref. 17). Therefore, CD8⁺ T-cell cross-priming might be critically dependent upon CD4⁺ T helper cell responses²⁶.

Concluding remarks

It has proven difficult to generate protective CD8⁺ T-cell immunity to microbes, viruses and cancer in humans with current vaccine strategies (e.g. peptides, plasmid DNA, subunit vaccines and inactivated viruses). One reason is that delivery of exogenous antigens to DCs has not been optimized. Cross-presentation allows us to consider approaches that exploit the special relationship between dead cells and DCs (e.g. *in vivo* delivery of DCs previously exposed to apoptotic tumor cells). These approaches are attractive as they can activate a broad repertoire of antigen-specific CD8⁺ and CD4⁺ T cells. Pilot clinical trials using DCs pulsed with tumor lysates suggest clinical efficacy^{64–66}. Many factors will have to be evaluated to optimize DC-based immunotherapy based on cross-presentation, such as the subset of DC, dosing, route of injection and optimal maturation signals. In doing so, a new appreciation of therapeutic concepts in vaccination will be revealed, not only in immune responses to pathogens and cancer but also to self-antigens.

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Opinion articles should present a personal viewpoint on a research-related topic, rather than a review of the topic. They can cover controversial areas that are being hotly debated, or present new models or hypotheses (along with suggestions for future experiments), or speculate on the meaning/interpretation of some new data, and should always stimulate debate. If you think you have an opinion that other immunologists should hear then e-mail a brief outline to:

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thanks!

the enhanced delivery of receptors from late endosomes to the apical cell surface. In contrast to these observations, we failed to increase the rate of transcytosis in MDCK cells transfected with human pIgR by raising the basolateral concentration of human pIgA 20-fold (Fig. 1)¹¹. Notably, at these concentrations of human ligand, the rate of transcytosis of rabbit pIgR was reported to be increased in a dose-dependent manner⁶.

This disparity between human pIgR and its rabbit and rat counterparts points to real species differences between IgRs, a fact not mentioned by van Egmond *et al.*¹. Although some spurious inability of human pIgR to associate with the MDCK signaling pathway cannot be excluded, this is not a probable explanation. Indeed, data are available to suggest that the species disparity is explained by the differing sensitivity of pIgR from various species to intracellular calcium, and not by the defective production of second messengers in the human test systems¹². Also, a substantial amount of human pIgR is constitutively transcytosed across secretory epithelial cells *in vivo* without any bound ligand^{5,6}. This observation is supported by the reported levels of total (bound and free) SC in secretions from hypogammaglobulinemic or IgA-deficient subjects; the level of free (plus SIgM-bound) SC in these patients is of the same magnitude as that of free (plus SIgA- and SIgM-bound) SC in normal controls^{5,6}. Thus, there is no *in vitro* or *in vivo* evidence to suggest that ligand-dependent stimulation of pIgR transcytosis takes place in humans.

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promoting protection than immature DCs. In this case, the protective response seems to be a T helper 2 (Th2) response.

Our studies in nonobese diabetic (NOD) mice, a murine model of autoimmune diabetes, have provided evidence that DCs are an attractive therapeutic option. However, the most effective type of DC might not be the same as that described for the induction of transplantation tolerance. In our experiments, we compared immature DCs with mature DCs in their ability to protect young, pre-diabetic NOD mice from diabetes and found that mature DCs were more effective, with immature DCs having little or no effect³. Subsequently, we showed that the therapeutic population of mature DCs expressed high levels of costimulatory molecules (e.g. CD80 and CD86) and produced low levels of IL-12 p70 protein following the ligation of CD40 (Ref. 4). This phenotype is compatible with the observation that therapy with DCs induced a Th2 response, as demonstrated by the induction of expression of islet-specific IgG1 antibodies³. Several other studies have demonstrated the effectiveness of therapy with mature DCs in the NOD mouse model^{5,6}, although the detailed phenotype and function of the populations of DCs used was not described in all cases.

Also, our results are supported by the description of defects in the number and function of DCs in rat and mouse diabetes models, as well as in pre-diabetic humans^{7–9}. It has been reported that the yield of DCs from monocytes was lower in pre-diabetic and diabetic humans than normal individuals, and that these DCs expressed lower levels of CD80 and CD86 (Ref. 8). In the NOD mouse, CD80⁺ DCs have been shown to express lower levels of CD86, which was associated with a reduction in the expression of cytotoxic T-lymphocyte Ag 4 (CTLA-4) following T-cell activation⁹. In addition, NOD mice have been shown to have a relative deficiency in the number of T_{reg} cells, which might be related to the defect in DCs. In a more recent study, B7^{-/-} NOD mice were shown to develop more aggressive diabetes than wild-type controls, and this was attributed to the lack of development of T_{reg} cells¹⁰. In this model, the development of CD4⁺CD25⁺ anergy-inducing T_{reg} cells¹⁰ appears to be dependent on the expression of high levels of costimulatory molecules. In addition,

How do dendritic cells prevent autoimmunity?

Two articles^{1,2} in recent issues of *Trends in Immunology* have argued that immature dendritic cells (DCs) can be considered 'regulatory' because they induce tolerance to allo- and auto-antigens (Ags). There is compelling evidence that immature DCs can induce either anergy or the development of regulatory T cells (T_{reg}) in the context of recognition of alloAg (reviewed in Refs 1,2). The studies described all demonstrate effectively that the naive immune system can be rendered tolerant using immature or interleukin-10 (IL-10)-modulated DCs. It has been suggested that similar approaches might be effective in the treatment of autoimmune diseases. However, the situation might be very different in this case because, in all probability, the autoimmune response will be ongoing at the time of therapy. We would like to draw attention to work performed by our group and others demonstrating that, in the case of autoimmune disease, DCs with a mature phenotype are more effective in

CD28^{-/-} NOD mice also developed more aggressive disease, and this was attributed to a defect in the development of Th2 cells¹¹. Although the evidence for the dependence of Th2-cell development on the expression of costimulatory molecules is somewhat contradictory, there are accumulating data suggesting that strong T-cell receptor interactions and a strong 'signal 2' (costimulation) are required. This type of stimulation is unlikely to be achieved by immature DCs, which express low levels of costimulatory molecules. Thus, we believe that by infusing NOD mice with DCs expressing high levels of CD80 and CD86 we might be correcting an intrinsic regulatory defect in these mice.

Although our experience concerns the diabetes model mainly, many autoimmune diseases are dominated by Th1 responses and therefore, the shift to a Th2 response would be advantageous. Thus, it is possible that strategies using mature DCs designed to stimulate Th2 responses will be effective in these conditions. A recent report demonstrated that DCs infected with IL-4-expressing adenovirus effectively prevented the onset of collagen-induced arthritis¹². Because it has been shown that adenoviral infection causes the maturation of murine DCs (Ref. 1), it is probable that the therapeutic effect in these studies is mediated by this mature population.

In conclusion, immature DCs are effective inducers of T_{reg} cells, which might function by secreting immunosuppressive cytokines, such as IL-10 and transforming growth factor β , but mature DCs might be necessary to induce protective Th2 responses, as well as anergy-inducing CD4⁺CD25⁺ suppressor T cells. It remains to be determined whether IL-10-modulated DCs or CD80⁺ DCs are effective in the treatment of autoimmune models. Thus, the choice of DC subtype for the induction of tolerance in autoimmunity might depend crucially on the nature of the ongoing autoimmune response.

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How do dendritic cells prevent autoimmunity: what is a mature dendritic cell in the mouse?

Response from Enk and Jonuleit

We appreciate the comment by Morel and Feili-Hariri with regard to our most recent publication in *Trends in Immunology*¹. We certainly do not argue that other subtypes of dendritic cell (DC) might not be suitable for the induction of tolerance also, but certain experimental differences should be considered, particularly with regard to the maturational status of the murine DCs, used by Feili-Hariri *et al.*^{2,3}

All of the studies mentioned by Morel and Feili-Hariri involve murine autoimmune models using bone-marrow-derived DCs that have been cultured in the presence of granulocyte–macrophage colony-stimulating factor (GM-CSF) – 'immature' DCs – or GM-CSF plus interleukin-4 (IL-4) – 'mature' DCs. Murine cultures (derived from bone-marrow stem cells) are much more heterogeneous than human cultures of DCs derived from CD14⁺ monocytes and the cells in the two types of culture are not identical. Indeed, it is questionable whether the maturational status of the cultures is comparable. Furthermore, as demonstrated in many murine and human studies, mature DCs normally induce the development of very efficient interferon- γ -producing T cells and their polarization into Th helper 1 cells *in vitro* and *in vivo*. Therefore, it should be noted that Feili-Hariri *et al.*^{2,3} use artificial autoimmune models for their studies which might not reflect a typical T-cell response to mature DCs (in a 'real' autoimmune disease).

We would also like to clarify a misunderstanding regarding the regulatory T cells described in the last paragraph of the letter by Morel and Feili-Hariri. Immature DCs do not induce regulatory T₁ (T_{reg}1) cells, but regulatory, CD4⁺CD25⁺ cytotoxic T-lymphocyte antigen 4⁺ T cells, which do not act by releasing IL-10, but by a contact-dependent mechanism. T_{reg}1 cells, as described by Roncarolo *et al.*⁴, clearly, are distinct from these cells. Furthermore, mature DCs are not able to induce the development of these CD4⁺CD25⁺ T_{reg} cells.

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Designer dendritic cells for tolerance induction: guided not misguided missiles

Holger Hackstein, Adrian E. Morelli and Angus W. Thomson

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that play crucial roles as initiators and modulators of adaptive immune responses. Although DC-based vaccines have been utilized successfully to generate cytolytic T-cell activity against tumor antigens (Ag), evidence has accumulated that DCs also have potent capabilities to tolerize T cells in an Ag-specific manner. DCs cultured in the laboratory can suppress auto- or alloimmunity. Current and prospective strategies to promote this inherent tolerogenic potential of DCs might prove to be important for the therapy of transplant rejection and autoimmune diseases.

Dendritic cells (DCs) play a dual role in the regulation of immune responses. For a long time, attention has been focused on the exceptional ability of these professional antigen-presenting cells (APCs) to elicit T- and B-cell-mediated responses, and their potential as therapeutic immunological adjuvants. However, a role for thymic DCs in central tolerance has been illustrated by their ability to delete self-reactive T cells¹. More recently, evidence has emerged concerning the inherent tolerogenicity of DCs in the periphery. Mechanisms whereby DCs might promote peripheral tolerance are under intensive investigation. These studies will have

important implications for the therapy of autoimmune disorders, allograft rejection and allergy.

The role of DCs in peripheral tolerance

DCs are extremely well equipped for their roles in innate and adaptive immunity. They are unrivaled in their ability to capture macromolecules, by macropinocytosis and mannose receptor-mediated endocytosis, into MHC class II-rich intracellular compartments². Following the uptake of antigen (Ag), activated DCs migrate from the periphery to secondary lymphoid tissue, where they redistribute MHC–Ag complexes to the cell surface and upregulate their surface expression of costimulatory molecules (e.g. CD80 and CD86) and others that promote the survival of DCs and DC–T-cell clustering [e.g. CD40, receptor activator of nuclear factor κB (RANK), CD54 and CD58]³. This process of maturation converts DCs into very powerful T-cell-priming APCs.

‘...how are DCs prevented from inducing an immune response against self-Ag?’

Given that immature DCs are disseminated throughout the peripheral tissues and capture Ag from dying cells during the turnover of normal tissue, the question arises: how are DCs prevented from inducing an immune response against self-Ag? Two publications have demonstrated that damaged cells are handled very differently by DCs, depending on how they die; necrotic cells promote the maturation of DCs and strong CD4⁺ and CD8⁺ T-cell stimulatory activity, whereas apoptotic cells fail to activate DCs (Refs 4,5). It could be argued that the uptake of apoptotic bodies in peripheral tissues is a ‘neutral’ process, which might not lead necessarily to the presentation of self-Ag to lymphoid tissue-resident T cells. However, this view has been challenged by Huang *et al.*, who demonstrated that

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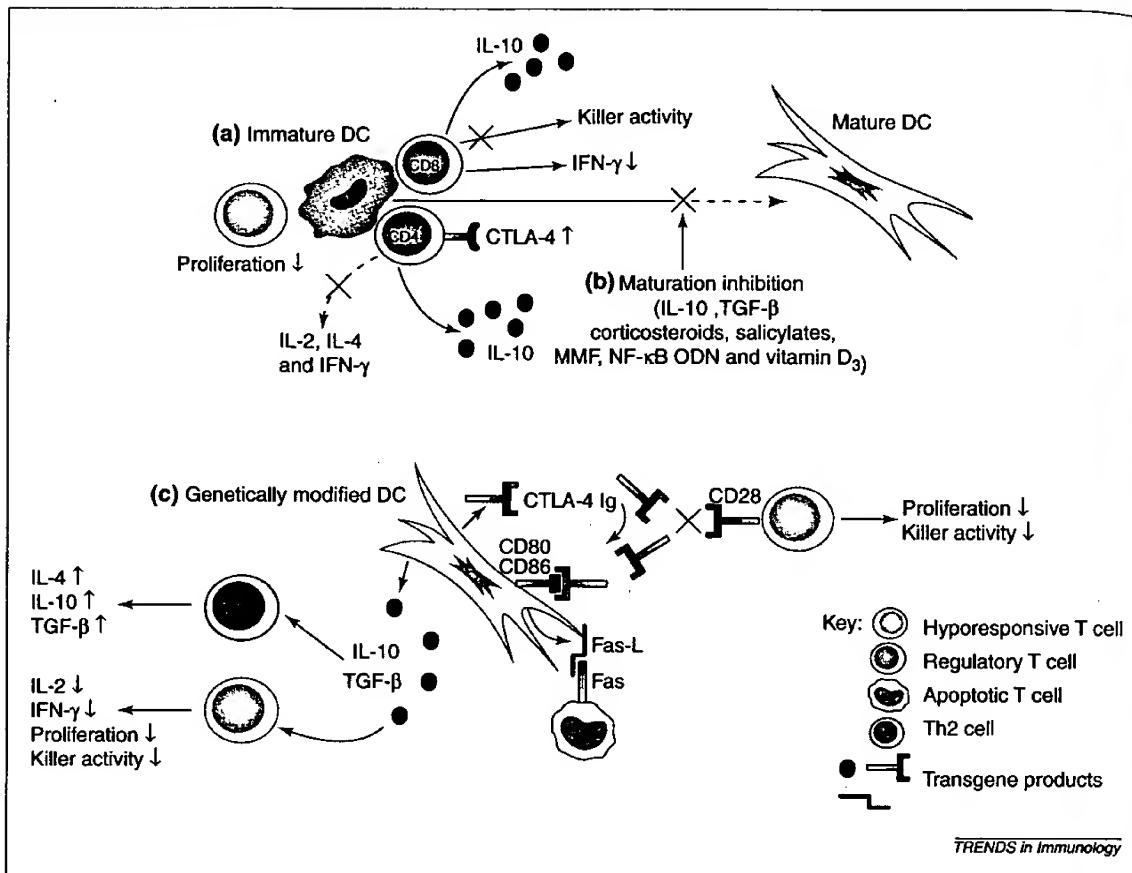


Fig. 1. Schematic representation of the mechanisms of downregulation of immunity. (a) Repetitive stimulation of T cells with allogeneic immature dendritic cells (DCs) results in suppressed proliferation of alloreactive T cells and leads to the generation of interleukin-10 (IL-10)-producing regulatory CD4⁺ T cells. Regulatory T cells upregulate their surface expression of the negative-regulatory cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) but do not produce IL-2, IL-4 or interferon γ (IFN- γ)¹². In human volunteers, the subcutaneous injection of autologous monocyte-derived DCs pulsed with influenza matrix peptide and keyhole limpet hemocyanin leads to the appearance of antigen (Ag)-specific CD8⁺ IL-10-producing regulatory T cells and the inhibition of Ag-specific T-cell killing activity, accompanied by a decrease in the number of IFN- γ -producing T cells¹³. (b) Pharmacological treatment – with cytokines [e.g. IL-10 (Ref. 18), transforming growth factor β (TGF- β)¹⁷], anti-inflammatory (e.g. corticosteroids¹⁴ and salicylates¹⁵) or immunosuppressive [e.g. mycophenolate mofetil (MMF)¹⁶] agents, oligodeoxyribonucleotides encoding nuclear factor κ B (NF- κ B)-binding sites (NF- κ B ODN)²², and 1 α ,25-dihydroxyvitamin D₃ (Ref. 19) – inhibits the maturation of DCs. (c) Genetic engineering of DCs to express the immunosuppressive molecules IL-10, TGF- β or CTLA-4 Ig promotes T-cell hyporesponsiveness^{24,25}, accompanied by reduced T-cell killing activity *in vitro*^{24,25} and *in vivo*²⁶, and T helper 2 (Th2)-skewing of anti-donor responses *in vivo*²⁶. T-cell apoptosis and subsequent Ag-specific hyporesponsiveness can be achieved by the genetic engineering of DCs to express Fas ligand (FasL)^{27,28}.

a subpopulation of rat intestinal DCs (CD11c⁺CD4⁻OX41⁻) constitutively transports apoptotic epithelial cells to the T-cell areas of mesenteric lymph nodes⁶. This intriguing report further questions the paradigm that DCs only migrate to draining lymph nodes when they have captured nonself-Ag in the context of inflammatory stimuli. The observations are in agreement with reports

showing that airway (lung)⁷, intestinal Peyer's patch⁸ or liver-derived⁹ DCs induce T helper 2 (Th2) responses preferentially, suggesting a view of DC-mediated tolerogenicity in the periphery that complements the classic view of immunostimulatory DCs.

One mechanism that might be used by DCs to maintain self-tolerance is the presentation of exogenous (primarily cell-derived) Ag to CD8⁺ T cells (cross presentation) or Th cells. Studies using transgenic animals expressing model self-Ag (hemagglutinin or ovalbumin) in peripheral tissues have provided evidence that CD4⁺ or CD8⁺ T-cell tolerance requires the presentation of cell-derived self-Ag by APCs (Refs 10,11). The term tolerance is used in different contexts in relation to the subversion of immune reactivity [e.g. Th2-skewing, T-cell anergy or deletion, or the induction of regulatory T (T_{reg}) cells]. For simplicity, we use the terms 'tolerance' and 'tolerogenic' as operational terms to indicate treatment modalities that induce the long-term survival of allografts or prevent and/or suppress clinically relevant autoimmune diseases in experimental animal models.

Current strategies to promote the tolerogenicity of DCs
Presently, two different approaches for the selective enhancement of the tolerogenic properties of DCs are under investigation (Fig. 1): (1) the use of immature DCs or the pharmacological arrest of the

Table 1. Treatment of allograft rejection and autoimmune diseases with DCs, without additional immunosuppression^a

DC type and modification	Application	Species	Effect	Refs
Allograft rejection				
GM-CSF-cultured immature myeloid donor DCs	i.v.	Mouse	Prolonged or indefinite heart allograft survival	29,30
GM-CSF-cultured liver-derived donor DC progenitors	i.v.	Mouse	Prolonged islet allograft survival	31
NF- κ B ODN-treated myeloid donor DCs	i.v.	Mouse	Prolonged heart allograft survival	23
FasL-transfected myeloid donor DCs	i.p.	Mouse	Prolonged heart allograft survival	28
Adenoviral IL-10- and/or TGF- β -transfected myeloid donor DCs	p.v.	Mouse	Prolonged kidney allograft survival	26
CTLA-4 Ig-transfected donor DC-line	i.v.	Mouse	Prolonged islet allograft survival	32
CD8 α^+ 'lymphoid-related' donor DCs	i.v.	Mouse	Prolonged heart allograft survival	38
Autoimmune diseases				
Adenoviral IL-4-transfected DCs	i.v.	Mouse	Suppression of collagen-induced arthritis	36
MBP-pulsed autologous myeloid DCs	s.c.	Rat	Prevention of MBP-induced EAE	33
GM-CSF-cultured (\pm IL-4 \pm islet-autoAg-pulsed) autologous myeloid DCs	i.v.	Mouse (NOD)	Decreased incidence of type 1 diabetes	34
Ex vivo IFN- γ -stimulated autologous splenic DCs	i.p.	Mouse (NOD)	Decreased incidence of type 1 diabetes	35

^aAbbreviations: Ag, antigen; CTLA-4 Ig, cytotoxic T-lymphocyte-associated antigen 4 Ig; DCs, dendritic cells; EAE, experimental allergic encephalomyelitis; FasL, Fas ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN- γ , interferon γ ; IL, interleukin; i.p., intraperitoneal; i.v., intravenous; MBP, myelin basic protein; MMF, mycophenolate mofetil; NF- κ B ODN, oligodeoxynucleotides encoding NF- κ B-binding sites; NOD, nonobese diabetic; p.v., portal venous; s.c., subcutaneous; TCR, T-cell receptor; Th, T helper; TGF- β , transforming growth factor β .

maturation of DCs; and (2) the use of genetically engineered DCs expressing immunosuppressive molecules. Jonuleit *et al.* have shown that repetitive *in vitro* stimulation of allogeneic human T cells with immature, monocyte-derived DCs leads to the generation of nonproliferating, interleukin-10 (IL-10)-producing T_{reg} cells¹². Proliferation of these T cells could not be restored by the addition of exogenous IL-2, and they inhibited the proliferation of Th1 cells in a contact-dependent, but Ag-nonspecific, manner.

'...NOTCH signaling drives the differentiation of peripheral CD4 $^+$ T cells into T_{reg} cells.'

The biological significance of these findings has been highlighted by Dhopakar *et al.*, who injected autologous, monocyte-derived immature DCs, pulsed with influenza matrix peptide and keyhole limpet hemocyanin, subcutaneously in two human volunteers¹³. They reported an Ag-specific inhibition of CD8 $^+$ T-cell killing activity and the appearance of peptide-specific IL-10-producing T cells, accompanied by a decrease in the number of interferon γ (IFN- γ)-producing T cells. Two remarkable findings emerged from these investigations. First, both studies associated the appearance of DC-induced T_{reg} cells

with the production of IL-10, but not IL-4. Second, the loss of T-cell killing activity was not owing to a decline in the number of peptide-specific T cells, indicating the importance of immune regulation rather than deletion of effector cells.

Thus, based on these findings, one approach to promote the tolerogenicity of DCs is to suppress their maturation (Fig. 1). Several anti-inflammatory (e.g. corticosteroids¹⁴ and salicylates¹⁵) or immunosuppressive (e.g. mycophenolate mofetil¹⁶) agents, as well as certain cytokines [e.g. transforming growth factor β (TGF- β)¹⁷ and IL-10 (Ref. 18)] and 1 α ,25-dihydroxyvitamin D₃ (Ref. 19) have been shown to inhibit the maturation of DCs. One limitation of these substances for the selective expansion of immature DCs *in vitro* is that some [e.g. corticosteroids¹⁴, 1 α ,25-dihydroxyvitamin D₃ (Ref. 19) and IL-10 (Ref. 20)] can profoundly inhibit the differentiation of precursor cells into DCs. Interestingly, aspirin promotes a relative increase in the number of immature bone-marrow-derived CD11c $^+$ DCs *in vitro*, and these cells do not induce a cell-mediated contact hypersensitivity response after subcutaneous injection in a mouse model¹⁵. Because the activation of nuclear factor κ B (NF- κ B) is crucial for the maturation of DCs (Ref. 21) – corticosteroids, salicylates and IL-10 inhibit the activation of NF- κ B (Ref. 22) – another approach to inhibit the maturation of DCs is the direct targeting of the activation of NF- κ B by employing

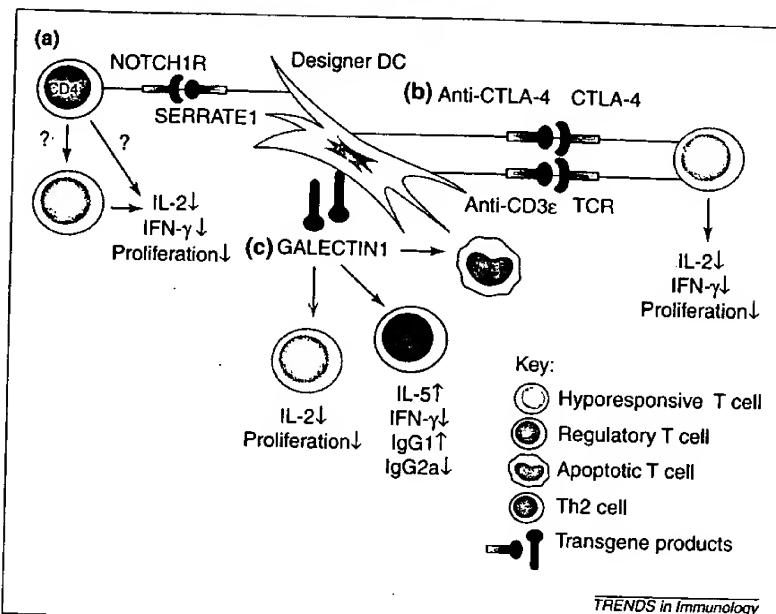


Fig. 2. Schematic representation of novel strategies to 'design' tolerogenic dendritic cells (DCs) by genetic engineering. (a) Serrate1-transfected DCs promote the generation of regulatory CD4⁺ T cells, presumably through engagement of the NOTCH1 receptor (NOTCH1R) on T cells⁴⁴. Immunization of animals with house dust mite peptide-pulsed, Serrate1-transfected DCs inhibited peptide-specific primary and secondary T-cell responses and was accompanied by the suppressed secretion of interleukin-2 (IL-2) and interferon γ (IFN- γ) by lymph node cells after *ex vivo* restimulation⁴⁴. Peptide-specific tolerance could be transferred to naïve animals by the injection of CD4⁺, but not CD8⁺, T cells from Serrate1-transfected-DC-treated animals⁴⁴. (b) The hypothetical induction of T-cell hyporesponsiveness by DCs transfected to express single-chain antibodies against cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and CD3e (Ref. 48). (c) The hypothetical impact of DCs engineered to express GALECTIN1, an immunosuppressive endogenous lectin. The promotion of T-cell apoptosis, T-cell hyporesponsiveness and Th helper 2 (Th2)-skewing are hypothesized on the basis of Refs 50,51.

oligodeoxyribonucleotides encoding NF- κ B-binding sites²³.

Genetic engineering of allogeneic DCs to express IL-10, TGF- β or the costimulation-blocking agent cytotoxic T-lymphocyte Ag 4 Ig (CTLA-4 Ig) induces Ag-specific T-cell hyporesponsiveness^{24,25}, inhibits the generation of cytotoxic T cells, both *in vitro*^{24,25} and *in vivo*²⁶, and promotes Th2-skewing of anti-donor responses²⁶ (Fig. 1). The selective hyporesponsiveness and apoptosis of Ag-specific T cells can be achieved by transfecting DCs with cDNA encoding Fas ligand (FasL)^{27,28} (Fig. 1).

In vivo treatment of experimental transplant rejection and autoimmune diseases with DCs

Evidence for the tolerogenic ability of DCs, either as immature cells or after *ex vivo* manipulation, comes from studies where DCs have been administered to immunocompetent recipients before transplantation, or models of autoimmune disease (Table 1). These studies have demonstrated that DCs of donor- or recipient-origin can prolong the survival of heart^{23,28-30}, kidney²⁶ or pancreatic-islet allografts^{31,32}. In addition, autologous DCs, with or without autoAg-pulsing, inhibit the development of experimental allergic encephalomyelitis (EAE)³³, type 1 diabetes^{34,35} and collagen-induced arthritis³⁶.

(Table 1). Interestingly, the majority of studies have focused on the administration of myeloid DCs. In mice, CD8 α ⁺ lymphoid-related DCs can inhibit the induction of T-cell reactivity by CD8 α ⁻ myeloid DCs (Ref. 37) and prolong the survival of heart allografts³⁸.

With the exception of one study involving the intravenous administration of donor DCs before transplantation²⁹, the permanent acceptance of organ allografts has not been achieved using DC therapy alone. Several investigators have used donor DCs in conjunction with treatment with immunosuppressive drugs. In particular, blockade of the CD40-CD154 signaling pathway, using anti-CD154 monoclonal antibodies, is synergistic with DC therapy³⁹. The value of blocking the CD40 pathway is demonstrated by the fact that this strategy promotes the survival of skin allografts, even in combination with Fms-like tyrosine kinase receptor 3 ligand (Flt3L)-expanded donor DCs (Ref. 40). By contrast, we found that with respect to the survival of a vascularized heart allograft, the calcineurin inhibitor tacrolimus did not show synergistic effects with Flt3L-mobilized donor DCs (Ref. 41). Flt3L is a hematopoietic growth factor that dramatically increases the *in vivo* generation of DCs, which when freshly isolated, are immature and potentially tolerogenic.

Solutions from novel strategies?

Although immature or genetically modified DCs prolong the survival of allografts and inhibit the development of EAE, type 1 diabetes and arthritis in different animal models (Table 1), it is currently difficult to achieve peripheral tolerance by DC-based immunotherapy. Three significant problems might account for this failure: (1) the approaches used might be unable to induce a sufficient number of T_{reg} cells required for the maintenance of tolerance; (2) the majority of transplant studies have focused on the use of donor DCs and therefore, target the direct pathway of allorecognition primarily, despite the fact that suppression of indirect allorecognition might be at least as important⁴²; and (3) current methods of gene-transfer to DCs might be either inefficient or lead to undesirable rather than desirable effects [for example, adenoviral vectors can induce the maturation of DCs (Ref. 43)]. In addition, the gene products investigated so far might not be adequate for the induction of tolerance. Therefore, it might be necessary to evaluate the use of novel genes in conjunction with improved gene-transduction strategies.

Induction of T_{reg} cells

Recently, it has been reported that NOTCH signaling drives the differentiation of peripheral CD4⁺ T cells into T_{reg} cells⁴⁴ (Fig. 2). NOTCH signaling is an evolutionarily conserved pathway that plays a fundamental role in cell-fate decisions through cell-cell interactions⁴⁵. Using retroviral-mediated gene transfer, Hoyne *et al.*⁴⁴ created DCs that

overexpressed SERRATE1, one of the ligands for NOTCH proteins. The vaccination of animals with Serrate1-transfected, house dust mite peptide-pulsed DCs, strongly inhibited the subsequent proliferation of T cells following immunization with house dust mite protein⁴⁴. Interestingly, the effect was Ag-specific, and Serrate1-transfected DCs also inhibited established immune responses against the model Ag. Further direct evidence for the generation of T_{reg} cells was provided by the transfer of CD4 $^{+}$ T cells from Serrate1-transfected DC-treated animals to naive animals; these cells strongly inhibited Ag-specific immune responses in the recipient animal. This promising approach awaits further confirmation in models of auto- or alloimmunity.

'A novel source for the generation of immature DCs is the directed differentiation of DCs from embryonic stem cells.'

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the host by direct allorecognition. Because the expansion of autologous DCs *in vivo* by the hematopoietic growth factor Flt3L enhances the induction of oral tolerance following feeding of protein Ag (e.g. ovalbumin)⁴⁷, the question of whether oral tolerance to allo- or autoAg can be potentiated in this manner is worthy of investigation.

Novel genes – novel methods

An alternative approach to the expression of immunosuppressive molecules by APCs is the delivery of genes encoding surface-linked single-chain antibodies to 'artificial' APCs. This strategy has allowed the selective expression of anti-CTLA-4 and anti-CD3e single-chain antibodies on the cell surface, and the subsequent Ag-specific downregulation of activation of CD4 $^{+}$ and CD8 $^{+}$ T cells⁴⁸ (Fig. 2). A novel source for the generation of immature DCs is the directed differentiation of DCs from embryonic stem cells⁴⁹. This approach generates stable, long-term DC cultures, which can be manipulated subsequently by viral or nonviral gene transfer. Novel candidate genes that have potential for the induction of tolerogenic DCs include Galectin1, an endogenous lectin that promotes T-cell apoptosis^{50,51} and Th2-skewing⁵¹ (Fig. 2), and OX2, a DC-surface Ag that suppresses Th1 responses⁵² (Fig. 2).

Concluding remarks

The quest for an ideal tolerogenic DC for the suppression of transplant rejection or autoimmune and allergic diseases has just begun. The 'design' of such DCs is still in its infancy. In the near future, a better understanding of the molecules and mechanisms normally employed by DCs to maintain peripheral tolerance should allow improved approaches to the generation of tolerogenic DCs. It is expected that, acting as 'guided missiles', such tolerogenic DCs fashioned in the laboratory will migrate to lymphoid organs, and encounter and specifically suppress Ag-specific T cells with high precision – a level of precision that at present, only the immune system can achieve.

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2003 Jan 15, 75(1):137-145
2002 Jan 15, 73 (1 suppl):S19-S22
2. Blood, 2003 Feb 15, 101(4):1439-1445
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thanks!

Dendritic cells and tolerance induction

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SUMMARY

Dendritic cells (DC) are widely accepted as the most potent antigen-presenting cells (APC), and considerable interest has been generated in their potential for the immunological therapy of cancer and infectious disease. Recently, however, a broader understanding of the phenotypic diversity and functional heterogeneity of DC has been acquired. Thus, in addition to having a role in central tolerance, DC are now regarded as potential modulators of peripheral immune responses. Harnessing this potential may offer a new approach to the immunosuppressive therapy of allograft rejection or autoimmunity. Here, the concept of 'tolerogenic' DC is placed in the context of rapidly accumulating new evidence of the diverse properties of these important APC.

Keywords dendritic cells tolerance antigen presentation

INTRODUCTION

Dendritic cells (DC), first described by Steinman & Cohn in 1973 [1], are a trace leucocyte population whose properties for antigen presentation and initiation of T cell-dependent immune responses are more developed and considerably more potent than other antigen-presenting cell (APC) types (reviewed in [2]). For many years investigators have been dependent upon the isolation of relatively small numbers of terminally differentiated DC from accessible tissue sites, such as human blood or mouse spleen [3,4]. The recent identification of *in vitro* conditions in which DC can be propagated from progenitor cells present in bone marrow or blood [5–7] has led to a surge in interest in the properties of these cells and their potential therapeutic applications. Amongst the most significant recent observations are those showing that tumour peptide-pulsed DC can be used to elicit antigen-specific protective anti-tumour immunity in mice [8] and that tumour antigen-pulsed autologous DC can elicit a clinically significant response in B cell lymphoma patients [9]. These findings have important implications for the use of DC in immunotherapy of cancer. In a separate context, donor-derived DC can be propagated from the bone marrow of spontaneously tolerant mouse liver allograft recipients [10]. In addition, DC have been identified as prominent components of the multilineage donor leucocyte microchimaerism observed in patients with long-established

organ transplants [11,12]. These observations suggest that DC may be important in the generation of stable allograft acceptance. The purpose of this short review is to highlight one aspect of the broadening perspective of DC heterogeneity and functional diversity—the ability of these cells to contribute to the induction of immunological tolerance.

IMMUNOSTIMULATORY PROPERTIES AND HETEROGENEITY OF DENDRITIC CELLS

DC were first described [1] as a novel cell type exhibiting stellate morphology when isolated from suspensions of mouse splenocytes. They have since been demonstrated as a ubiquitous cell population in virtually all tissue sites examined, except central cornea and brain (reviewed in [2]). Whilst the exact developmental pathway of different DC populations is still poorly defined, *in vitro* and *in vivo* studies have confirmed the bone marrow origin of these leucocytes [13,14]. While phenotypic studies and the *in vitro* propagation of DC from progenitor cells in blood and bone marrow indicate that most DC populations are closely related to macrophages, they nevertheless constitute a distinct cell lineage [5,15]. DC do not typically express lineage-associated cell surface molecules found on lymphocytes, granulocytes or natural killer (NK) cells. CD4 and CD8, however, are expressed on certain subpopulations of DC [3,4]. Despite expressing many surface markers also associated with mononuclear phagocytes, DC constitutively express high levels of surface MHC class II gene products. This feature is often used in conjunction with functional characteristics to definitively characterize DC [2].

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It is now recognized that considerable heterogeneity exists between DC populations located in distinct tissue sites. While such variations may reflect differing developmental pathways [6,16,17], much of the heterogeneity appears to be related to the progression of DC through discrete stages of their life cycle. Circulating MHC class II-negative DC precursors migrate into non-lymphoid tissues and mature *in situ* to establish and maintain DC populations both in the steady state and during inflammation [18,19]. DC resident in peripheral tissue sites are specialized for antigen acquisition and processing and accordingly possess Fc receptors, lysosomal enzymes, and express some macrophage-associated markers [20,21]. Characteristically, when freshly isolated from peripheral tissue DC possess low levels of costimulatory molecules and exhibit a weak capacity to stimulate the activation of resting T cells *in vitro* [20,22]. Accumulating evidence indicates that DC in lymphoid tissues are derived, at least in part, from peripheral DC populations which have migrated to regional lymphoid tissue to complete their life cycle [23,24]. Therefore, peripheral DC are considered to function as sentinels of the immune system which can be mobilized to migrate to regional lymphoid tissues following contact with antigens or inflammatory stimuli [25,26].

Following emigration from peripheral tissue sites and migration to regional lymphoid tissues DC, typified by epidermal Langerhans cells (LC), undergo a process of phenotypic change and 'maturation' in which the phagocytic and endocytic capacity observed in freshly isolated cells is lost. At the same time there is a marked up-regulation in the expression of surface MHC class II [20,27-29]. In addition to these phenotypic changes, surface markers associated with antigen uptake, for example CD16/32, are lost and adhesion molecules and molecules associated with activation, for instance CD25, CD40, CD54 (intercellular adhesion molecule-1 (ICAM-1)), CD80 (B7-1) and CD86 (B7-2), are expressed at increased levels [30,31]. These changes are accompanied by a transformation to cells with a low buoyant density that possess a potent capacity to stimulate naive T cells. This latter property is considered characteristic of DC in lymphoid tissues [28,32] and is related to the increased expression of cell surface costimulatory molecules [30]. Within lymphoid tissues, DC accumulate in the T cell-dependent regions where they are ideally situated to interact with recirculating T cells [24,33] and select those with specific reactivity for the presented antigens. Examination of the functional capacity of APC isolated from secondary lymphoid tissues has indicated that these 'mature' DC

are the most potent stimulators of resting T cells, with a capacity which exceeds that of other APC by up to 1000-fold [32,34]. Studies employing antigen-pulsed DC have additionally indicated the capacity of these cells to stimulate primary antigen-specific responses *in vivo* is at least 100-fold that of other APC types [35,36]. All of these features meld to impart DC with a pivotal role as sentinels of the immune system responsible for the induction of primary T and possibly B cell responses (reviewed in [2]).

It has generally been regarded that DC present as 'passenger leucocytes' in transplanted organs provide the primary stimulus for allograft rejection [37-39]. The liver, which is the most leucocyte-rich of transplanted organs, is however widely recognized as the least immunogenic and can protect other organs from rejection [40]. A recent clue to the mechanisms of tolerogenicity exerted by hepatic allografts are reports that leucocytes of donor origin, of which DC feature prominently, can be identified at distant sites of long-surviving successful graft recipients [11,12]. This has given rise to the idea that donor-derived cells (donor leucocyte microchimaerism) may be a cause rather than a consequence of graft survival, and that evolving mutual suppression of donor-recipient leucocyte interaction may be the basis of transplantation tolerance (the two-way paradigm) [41-43].

THE BROADENING PERSPECTIVE OF DC FUNCTION: THEIR ROLE IN TOLERANCE INDUCTION

Whilst extensive studies have resulted in a greater understanding of the critical role of DC in the stimulation of primary immune responses, evidence has also accumulated that the activity of DC is not restricted to such a narrow niche within the immune system. Indeed these cells, which are the most effective activators of resting/naive T cells, also appear to regulate the nature of immune responses through the shaping of tolerance induction.

The thymus plays a major role in immune homeostasis by the deletion of self-reactive T cells, thus contributing to the maintenance of self-tolerance [44]. A number of cell types present within the thymus may potentially provide signals responsible for the negative selection of developing T cells (reviewed in [45]). However, two lines of evidence suggest that a substantial proportion of this activity is provided by thymic

Table 1. Observations of the tolerogenic capacity of dendritic cells (DC)

Dendritic cell type	Tolerogenic effect reported	Reference
Thymic DC	Encephalitogen pulsing and adoptive transfer prevents EAE	[58]
	Tolerance acquired to host MHC in chimaeric thymi	[46]
Langerhans cells	Depletion enhances effector phase of CH <i>in vivo</i>	[66]
Pancreatic lymph node DC	Transfer reduces incidence of diabetes in NOD mice <i>in vivo</i>	[62]
Splenic DC	Reconstitution of DC-depleted thymuses restores ability to delete thymocytes <i>in vivo</i>	[49]
	Large numbers of DC reduce local HVG reaction <i>in vivo</i>	[64]
	Large numbers of DC/high antigen load inhibit anti-tumour immunity <i>in vivo</i>	[63]
Costimulator-deficient DC progenitors	CD8 ⁺ /Fas-L ⁺ DC induce apoptosis in activated T cells <i>in vitro</i>	[81]
	Induction of alloantigen-specific unresponsiveness <i>in vitro</i>	[87]
	Adoptive transfer prolongs allograft survival <i>in vitro</i>	[88,89]

EAE, Experimental allergic encephalomyelitis; CH, contact hypersensitivity; HVG, host *versus* graft; NOD, non-obese diabetic.

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DC. When T cells differentiate in MHC-disparate thymus grafts depleted of bone marrow-derived APC, these T cells show only limited tolerance to the MHC antigens of the grafted thymuses [46-48]. Matzinger & Guerder [49] outlined the critical role of DC in central tolerance by demonstrating that the tolerogenic properties of the APC-depleted thymi could be restored by reconstitution with purified splenic DC. In this system, DC showed the strongest capacity to reconstitute tolerogenic activity; purified splenic DC were 100-fold more effective than unseparated spleen cells and 1000-fold more effective than thymocytes [49]. Similar results have been reported in parent to F₁ bone marrow chimaeras and in transgenic mice [50,51]. These findings indicate that the role of DC in deleting potentially autoreactive developing T cells in the thymus is not dependent upon unique characteristics of the thymic DC, but may be mediated by signals provided by other DC types. Intrathymic inoculation of exogenous antigens has additionally demonstrated that tolerance generated by T cell depletion within the thymus is not restricted solely to MHC antigens [52,53]. More recent studies directed at investigating the possible amelioration of autoimmunity or allograft rejection have indicated that the tolerance exhibited following intrathymic inoculation of exogenous antigen is dependent upon thymic DC [54,55]. Studies of lipopolysaccharide (LPS)-treated mice have suggested that increased apoptosis of thymocytes in these animals may be associated with nitric oxide (NO) production by presumptive thymic DC [56]. These findings are consistent with our own observations of NO production by DC following treatment with interferon-gamma (IFN- γ) or LPS [57].

While central tolerance may reflect the unique nature of the intrathymic environment or of T cell development, more recent studies suggest that tolerance induction mediated by DC is not confined to the intrathymic compartment (Table 1). Protection from autoimmunity induced by the encephalitogenic autoantigen myelin basic protein (MBP) can be achieved by i.v. injection of thymic DC either pulsed with the immunodominant peptide of MBP or isolated from thymi inoculated *in vivo* with MBP [58]. This protection was similar to that invoked by direct intrathymic inoculation of MBP [58]. The ability to induce tolerance is not restricted to thymic DC, as i.v. administration of antigen-pulsed LC or splenic DC has been reported to result in antigen-specific suppression of DTH responses [59,60], possibly via a mechanism involving selective activation of T helper type-2 (Th2)-like T cell subsets [61]. A further illustration of the ability of DC to shape a protective effect against autoimmunity has been provided by the demonstration that DC isolated from pancreatic lymph nodes of non-obese diabetic (NOD) mice and transferred to pre-diabetic NOD mice limited the expression of diabetes [62], possibly via a mechanism involving the enhanced induction of regulatory T cells.

While the *in vivo* administration of DC via particular routes, or of certain DC populations, has resulted in the generation of tolerance or certain tolerance-like states, it is likely that the nature of ongoing immune responses is dependent on signals provided by DC. This suggestion is consistent with observations that DC exhibit a dichotomous potential to regulate immune responsiveness. Thus *in vivo* experimental studies have indicated that administration of DC loaded with low doses of tumour antigen can enhance anti-tumour immunity, while administration of DC loaded with high doses of tumour antigen or administration of high numbers of tumour antigen-pulsed DC inhibit the development of protective anti-tumour immunity [63]. Interestingly, a similar phenomenon has been reported where inoculation of high numbers of semi-allogeneic DC reduced the expression of host-*versus*-graft reaction in a local *in vivo* assay [64]. Despite the role of LC in the induction phase of contact sensitivity [65], depletion of LC from epidermal surfaces of mice has been reported to result in markedly stronger expression of the effector phase of contact hypersensitivity, suggesting these cells may provide both stimulatory and down-modulatory signals [66].

It is possible that DC from specific peripheral tissue sites may possess the capacity to induce forms of tolerance whereby certain aspects of immune responsiveness are selectively inhibited while others remain intact. Considerable evidence has accumulated that an APC bearing an antigen-specific signal migrates from the eye following administration of soluble antigen into the anterior chamber (AC) and results in the activation of regulatory T cells in the spleen. It is proposed that this phenomenon accounts for aspects of the 'immune-privilege' of the AC (reviewed in [67]), apparently via the selective activation of a Th2-type response [68]. Recent work examining the APC populations present in the tissues surrounding the anterior chamber has provided a strong indication that the APC which migrates from the anterior chamber is an MHC class II-positive DC [69,70]. It is likely that specific components of the local tissue microenvironment, for example the cytokine transforming growth factor-beta (TGF- β) [67], are responsible for imparting DC with such properties, but the role of various other microenvironmental influences on DC function is yet to be clearly defined. Examination of other tissue sites may help to clarify this. There is also evidence that expression of immune unresponsiveness observed following application of antigen to mucosal surfaces (reviewed in [71,72]) is the result of selective activation of T cell subsets during primary immune stimulation. Whether DC from these tissue sites provide signals which result in the selective activation of T cell subsets is not yet determined, but ongoing studies may help to elucidate the immunoregulatory role of these DC.

Table 2. Experimental manipulations rendering tolerogenicity to dendritic cells

Dendritic cell type	Tolerogenic effect reported	Reference
CTLA4-Ig-treated splenic DC	Protection from EAE	[90]
IL-10 treated Langerhans cells	Antigen-specific anergy induction <i>in vitro</i>	[74]
Ultraviolet-irradiated Langerhans cells	Antigen-specific unresponsiveness of Th1 cells <i>in vitro</i>	[73]

EAE, Experimental allergic encephalomyelitis.

Experimental modulation of DC function, for example by exposure to ultraviolet (UV) irradiation or to the immunomodulatory effects of IL-10 (Table 2), has also been reported to induce antigen-specific hyporesponsiveness *in vitro* [73,74]. The relevance of such *in vitro* experimental treatments to the *in vivo* function of DC in conditions such as the tolerance that may be observed to UV-induced tumours is unclear. Interestingly, evidence is emerging that alteration of the immunostimulatory capacity of DC by the cytokine milieu within tumours [75] may contribute to the tolerance often observed with many tumour types [76]. Therefore the ability to manipulate the immunostimulatory capacity of DC is likely to further our understanding of immune regulation and lead to potential therapeutic strategies to enhance tumour clearance and/or allograft acceptance.

MOLECULAR REGULATION OF THE IMMUNOLOGICAL ACTIVITY OF DC

Studies examining the phenotypic characteristics of DC isolated from thymus and spleen of mice have demonstrated the presence of a major DC subpopulation in these tissues expressing significant levels of CD8 [77,78], comprised predominantly of the CD8 α homodimer [78]. It has been proposed that expression of the CD8 molecule by APC may provide cells with a 'veto' function, enabling them to inactivate T cell precursors with which they interact [79,80]. Expression of this molecule by major subpopulations of thymus and spleen DC may allow these cells to perform a regulatory role in immune responsiveness by both presenting antigen and inactivating T cells [78]. While experimental evidence is yet to be provided confirming the role of CD8 $+$ DC as veto cells, further work has demonstrated that the CD8 $+$ population of murine splenic DC express CD95 (FasL) and that these cells are capable of killing activated CD4 $+$ T cells via Fas-FasL interactions [81].

The elucidation of the role of costimulation in T cell activation (reviewed in [82]) has provided insight into one potential mechanism by which 'tolerogenic APC' may function. Our current understanding indicates that presentation of antigen to T cells by APC in the absence of essential costimulatory signals induces a state of antigen-specific unresponsiveness (anergy) in these T cells [83], and thus also tolerance. Such a proposal leads to speculation that DC in peripheral tissue sites, which exhibit low levels of costimulatory molecule expression in the steady state [30,31], may play a potentially important role in peripheral tolerance. Such a scheme would allow peripheral DC populations to perform a dual role, by contributing to the maintenance of peripheral tolerance in the steady state, and also by acting as effective sentinels following activation signals, for example, by microbial products (reviewed in [84]). While experimental studies are yet to provide evidence, MHC class II-negative DC precursors described in the airway epithelium of adult rats [19], which are poor allostimulators [85], and LC precursors described in the epidermis [86] may represent examples of endogenous populations of peripheral DC which possess the potential to function in such a manner.

Recent studies support the notion that DC deficient in costimulatory signals may effect tolerance induction. Thus DC progenitors propagated *in vitro* from mouse bone marrow in the presence of low concentrations of granulocyte-macrophage colony-stimulating factor express low levels of

costimulatory molecules (B7-1 low , B7-2 $^{-}$) and induce alloantigen-specific unresponsiveness (anergy) in T cells *in vitro* [87]. The alloantigen-specific unresponsiveness of T cells induced by *in vitro* propagated costimulator molecule-deficient DC is not restricted to *in vitro* assays, and administration of these cells or costimulatory molecule-deficient DC propagated from the liver under similar conditions has been demonstrated to prolong cardiac and pancreatic islet allograft survival, respectively [88,89]. Likewise, DC on which the activity of surface B7-1 and B7-2 costimulatory molecules has been blocked by a cytotoxic T lymphocyte-associated molecule-4 immunoglobulin fusion protein (CTLA4-Ig) have been demonstrated to protect animals from the induction of experimental autoimmune encephalomyelitis (EAE) [90]. It has also been suggested that rhesus monkey bone marrow-derived DC progenitors (MHC class II dim , CD8 $^{+}$) may exert tolerance-promoting activity both *in vitro* and *in vivo* [91].

CONCLUSION

While the extent to which 'tolerogenic' DC may regulate immune responsiveness is yet to be elucidated, there is sufficient justification for future studies aimed at evaluating the potential of these cells for therapy of allograft rejection and autoimmunity. Further understanding of the factors that regulate DC development and the outcome of DC-T cell interactions may provide targets for intervention. One approach which may prove fruitful is genetic engineering. Conceivably, the expression and/or secretion of immunosuppressive molecules by DC during their interaction with T cells, either during primary sensitization or ongoing *in vivo* responses, may enhance their tolerogenic capacity.

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RAPAMYCIN IMPAIRS ANTIGEN UPTAKE OF HUMAN DENDRITIC CELLS¹

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Background. Rapamycin is a recently introduced immunosuppressive agent. Its effect on lymphocytes has been extensively studied. Whether it can also modulate dendritic cell (DC) function is unknown.

Methods. The effect of rapamycin on differentiation, antigen uptake, and the immunostimulatory capacity of human DC was examined. DC were derived from monocytes upon culture with interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor in the presence or absence of rapamycin (0.1–100 ng/mL). Surface phenotype and antigen uptake capacity of DC were assessed by flow cytometry. Immunostimulatory capacity was measured by mixed lymphocyte culture.

Results. Rapamycin reduced DC recovery and increased DC apoptosis. DC differentiated in the presence of rapamycin (rapa-DC) had increased expression of CD1a, CD1b, and CD1c and decreased expression of MHC I, MHC II, CD80, CD86, and CD40. Antigen uptake receptor expression (mannose receptor, CD32, CD91, CD46) was decreased, and receptor-mediated endocytosis of fluorescein isothiocyanate-dextran was markedly impaired in rapa-DC, as were fluid phase endocytosis of Lucifer Yellow and phagocytic activity of bacteria and dead or apoptotic cells. CD40 ligand-induced production of both IL-12 and IL-10 was reduced in rapa-DC, and allogeneic T lymphocyte responses were moderately impaired when rapa-DC were used as stimulator cells. Neither cyclo-

sporine nor FK506 affected DC function. However, the effects of rapamycin on DC could be completely inhibited by a 10-fold excess of FK506 but not by up to 100-fold excess of cyclosporine.

Conclusion. Rapamycin has a unique and profound inhibitory effect on DC function, which seems to be at least in part mediated by the FKBP immunophilins.

Rapamycin is a macrocyclic triene antibiotic produced by the actinomycete *streptomyces hygroscopicus* (1). Although rapamycin was originally isolated for its antifungal properties, it is now used in clinical transplantation as a result of its efficacy in prolonging allograft survival in various animal models (2,3) and its unique mechanism of action (4–8). Structurally, rapamycin resembles tacrolimus (FK506) and binds to the same intracellular binding protein immunophilin, known as FKBP-12 (8). However, rapamycin has a novel mechanism of action. Although tacrolimus and cyclosporine block lymphokine (e.g., interleukin [IL]-2) gene transcription, rapamycin acts later to block IL-2-dependent T lymphocyte proliferation (4,8). It also inhibits IL-2-dependent and IL-2-independent proliferation of B lymphocytes and production of immunoglobulin A, immunoglobulin M, and immunoglobulin G (4–6). Like other immunosuppressive drugs used in allogeneic transplantation medicine, rapamycin has been studied extensively regarding its effects on T lymphocytes, but its effects on dendritic cells (DC) are relatively unknown. DC are the most potent antigen-presenting cell (APC) in vitro and in vivo, with a key role in the initiation of the immune response (9–13). With respect to transplantation, donor DC transplanted with the allograft migrate to the recipient spleen where they can activate naive T cells, thereby inducing an immune response through the direct pathway of allorecognition. Several studies showed that preventing the direct pathway of allorecognition by DC depletion resulted in prolonged graft survival. In addition, both transplant injury and local inflammation will attract recipient DC to the graft, and this contributes to the indirect pathway of antigen presentation. Therefore, DC might be an important target for immunosuppression to prevent allograft rejection

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and to induce transplant tolerance (14,15). The aim of this study was to determine whether rapamycin altered human DC differentiation, maturation, and functional activities. Our results demonstrate that rapamycin does not alter differentiation but does affect DC function by inhibiting antigen uptake capacity and maturation into potent APC. These effects seemed to be mediated by FKBP-12. These findings show a novel target of rapamycin action on the immune system and may have relevance in the development of new therapeutic treatments in the field of transplantation and autoimmune disease.

MATERIALS AND METHODS

Cytokines and Reagents

Human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF; specific activity 1.1×10^4 U/mg) was obtained from Novartis (Basel, Switzerland). Human recombinant IL-4 (specific activity $>2 \times 10^6$ U/mg) was from Pepro Tech EC LTD (London, England). Rapamycin (sirolimus) was from Sigma (St. Louis, MO). Cyclosporine (CsA) was from Sandoz Pharma Ag (Basel, Switzerland). Daclizumab (anti-CD25 monoclonal antibody) and mycophenolate mofetil (MMF) were from Roche Laboratories (Nutley, NJ). Tacrolimus (FK506) was from Fujisawa Pharmaceutical (Osaka, Japan).

DC Culture

Highly enriched monocytes ($>95\%$ CD14 $^{+}$) were obtained from buffy coats of blood donors (through the courtesy of Centro Trasfusionale, Ospedale San Raffaele, Milan, Italy) by Ficoll and Percoll gradients and purified by adherence. Monocytes were cultured for 7 days at 10^6 /mL in 6-well multiwell tissue culture plates (Falcon, Becton Dickinson, NJ) in RPMI (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 50 ng/mL GM-CSF, and 10 ng/mL IL-4, and with or without different concentrations of rapamycin, CsA, FK506, or MMF. Under control conditions (GM-CSF + IL-4), the cell yield was about 80% of input cells. All the cultures were tested for the presence of endotoxin (<0.03 U/mL; Lymulus Test).

DC Maturation

Lipopolysaccharide (LPS; 10 ng/mL) was added to induce maturation of DC for at least 36 hr of culture. Alternatively J558L cells transfected with the ligand for CD40 (J558LmCD40L) were used to induce CD40 triggering on DC. Untransfected J558L cells were used as control. After irradiation (10,000 rad), J558L cells were seeded together with DC at a 1:1 ratio in 24-well culture plates in culture medium (10^6 cells/well). Cells were recovered after 48 to 72 hr of culture.

Fluorescence-Activated Cell Sorter (FACS) Analysis

Cell staining was performed using mouse monoclonal antibodies followed by fluorescein isothiocyanate (FITC)-conjugated affinity-purified, isotype-specific goat anti-mouse antibodies (Ancell, Bayport, MN). The following monoclonal antibodies were used: L243 (IgG2a anti-MHC class II), 32.2 (anti-CD32), OKT4 (IgG2b, anti-CD4), OKT3 (Ig2a, anti-CD3) from American Type Culture Collection (Rockville, MD); UCHM-1 (IgG2a, anti-CD14) and W6/32 (IgG2a, anti-MHC I) from Sigma; SK9 (IgG2b, anti-CD1a) from Becton Dickinson (San Jose, CA); WM25 (IgG1, anti-CD1b), PHM-3 (IgG2a, anti-CD1c), and Nor 3.2 (IgG1, anti-CD1d) from Cymbus Biotechnology LTD (Chandlers Ford, Hants); 122-2 (IgG1, anti-CD46) from Chemicon (Temecula, CA); A2Mr alpha-2 (IgG1, anti-CD91) from Serotec (Kidlington, Oxford, UK); B73.1 (IgG2a, anti-CD16), kindly provided by Dr. P. Allavena, Milan, Italy; PAM-1 (IgG1, anti-mannose receptor; 16), kindly provided by Dr. Biondi,

Milan, Italy; BB1 (IgM, anti-CD80), BU63 (IgG1, anti-CD86), and EA-5 (IgG1, anti-CD40) from Ancell; and HB15a (IgG2b, anti-CD83), B1.49.9 (IgG2a, anti-CD25), and CF-1 (IgG1, anti-CD122) from Immunotech (Marseille, France).

Results are expressed as percentage of positive cells or as fluorescence intensity (FI), calculated according to the formula: FI = mean fluorescence (sample) - mean fluorescence (control).

Endocytosis and Phagocytosis

Mannose receptor-mediated endocytosis was measured as the cellular uptake of FITC-dextran and quantified by flow cytometry. Approximately 2×10^6 cells per sample were incubated in media containing FITC-dextran (1 mg/mL) (molecular weight 40,000; Sigma) for 0, 60, and 120 min. After incubation, cells were washed twice with phosphate-buffered saline (PBS) to remove excess dextran and fixed in cold 1% formalin. The quantitative uptake of FITC-dextran by the cells was determined using flow cytometry. At least 8000 cells per sample were analyzed. Fluid phase endocytosis through membrane ruffling was measured as the cellular uptake of 1 mg/mL of Lucifer Yellow Dipotassium Salt (Sigma) and quantified by flow cytometry. Phagocytic activity was evaluated as uptake of *Staphylococcus aureus* with or without opsonization, as internalization of green fluorescent beads (1.2 μ m), and as internalization of apoptotic or necrotic fluorescent-labeled Jurkat cells. *S. aureus* BioParticles, opsonizing reagent, and FluoSpheres beads were obtained from Molecular Probes (Eugene, OR). *S. aureus* BioParticles, either untreated or after opsonization, were incubated with control DC (ctrl-DC) or rapamycin-treated DC (rapa-DC) for 30 min at 4°C. Binding to DC membrane was evaluated by FACS Scan (Becton Dickinson) and expressed as percentage of positive cells or mean fluorescence intensity. Particle uptake was evaluated by incubating *S. aureus* BioParticles or FluoSpheres beads with ctrl-DC or rapa-DC for 120 min at 37°C or 4°C.

After phagocytosis, DC were washed and then incubated in PBS containing EDTA and trypsin for 15 min at 37°C to eliminate membrane-bound particles. Cells were washed and analyzed by flow cytometry and microscopy. Jurkat cells were committed to apoptosis by 72 hr of culture without serum. Apoptosis was verified by flow cytometry and by morphologic features. Necrosis was achieved by freezing and thawing cycles. Jurkat cells were labeled with the green fluorescent aliphatic dye PKH2-GL (Sigma) and then coincubated with ctrl-DC or rapa-DC for 120 min at 37°C or 4°C. After phagocytosis, DC were washed and then incubated in PBS containing EDTA and trypsin for 15 min at 37°C to eliminate membrane-bound particles. Then the cells were washed and analyzed by flow cytometry. Phagocytic activity was expressed as percentage of positive cells (calculated as positive cells at 37°C - positive cells at 4°C) and mean fluorescence intensity of positive cells (calculated as mean fluorescence intensity of positive cells at 37°C - mean fluorescence intensity of positive cells at 4°C).

Mixed Leukocyte Reaction (MLR) and Priming of Allogeneic Naive T Cells

DC cultured in GM-CSF + IL-4 with or without rapamycin for 7 days were extensively washed, irradiated (3000 rad from a 137-Cs source) and added in graded doses to 10^6 responder cells in 96-well flat-bottomed microtest plates (Costar Corp., Cambridge, MA). Responder cells were purified allogeneic T cells depleted of autologous APC by passage with CD14- and CD19-coated Dynabeads (Unyphat, Milan, Italy). Each group was performed in triplicate. Thymidine incorporation was measured on day 5 by a 16-hr pulse with 3 H-thymidine (1 mCi/well, specific activity, 5 Ci/mMol; Amersham Life Science, Buckingham, UK). Naive T cells, obtained from cord blood, were cultured a ratio of 10:1 with allogeneic ctrl-DC or rapa-DC. After 5 days, proliferating cells were expanded with IL-2 and analyzed between day 8 and 14. For cytokine detection at the single-cell level, the following monoclonal antibodies were used: MP4-25D2

(rat IgG1, anti-IL-4) from Serotec and G7-4 (mouse IgG, anti-interferon [IFN]-gamma) from Bender MedSystems.

Apoptosis and Metabolic Activity Detection

Phosphatidylserine exposure was determined using an annexin V-FITC Kit (Bender MedSystems) in combination with propidium iodide (PI; molecular probes). Cells were harvested, washed, labeled with annexin V-FITC for 30 min on ice, and subsequently taken up in 1 mg/mL PI. Annexin V-PI staining was analyzed on a FACScan using Cell Quest software. Metabolic activity of DC was evaluated by using the alamarBlue assay. Reduced activity because of cell growth or viability causes the REDOX indicator to change from the oxidized (nonfluorescent, blue) form to the reduced (fluorescent, red) form. AlamarBlue is added to cell cultures at the final dilution of 1:10 (incomplete medium) and incubated at 37°C for 6 to 18 hr. Relative absorbance was read by a Cytofluor 2350 (Millipore, Bedford, MA) at 530 to 590 λ .

IL-10 and IL-12 p70 Assays

After 7 days of culture with GM-CSF (50 ng/mL) and IL-4 (10 ng/mL) in the presence or absence of rapamycin, DC were stimulated with CD40L or LPS for 48 hr. Medium was collected and cytokines were quantified by ELISA (IL-10, IL12 p70, IL-1 β) with a commercial kit by Pierce Endogen (Woburn, MA).

RESULTS

DC Phenotype

To investigate the effect of rapamycin on DC differentiation from monocytes, we cultured monocytes in the presence of GM-CSF, IL-4 (control group; ctrl-DC), and different concentrations (0.1–10 ng/mL) of rapamycin (rapa-DC). Upon culture with GM-CSF and IL-4, the cells became nonadherent and clustered and had abundant cytoplasm and protruding veils typical of DC. DC in the presence of rapamycin showed a similar morphology (Fig. 1A). Cell recovery was 81±5%, 79±16%, and 64±14% of ctrl-DC for 0.1, 1, and 10 ng/mL of rapamycin, respectively. Rapamycin-reduced cell recovery (Fig. 1B) was a result of apoptosis as confirmed by either annexin V-PI staining or microscopy (Fig. 1A and D). The presence of rapamycin during differentiation of DC from monocytes induced modification in phenotype (Table 1). Rapa-DC expressed higher levels of CD1a, CD1b, and CD1c than ctrl-DC. Both ctrl-DC and rapa-DC appeared negative for CD1d expression. MHC class I, MHC class II, CD40, CD86, and CD14 expression was decreased. DC obtained after 7-day culture with GM-CSF and IL-4 could be further

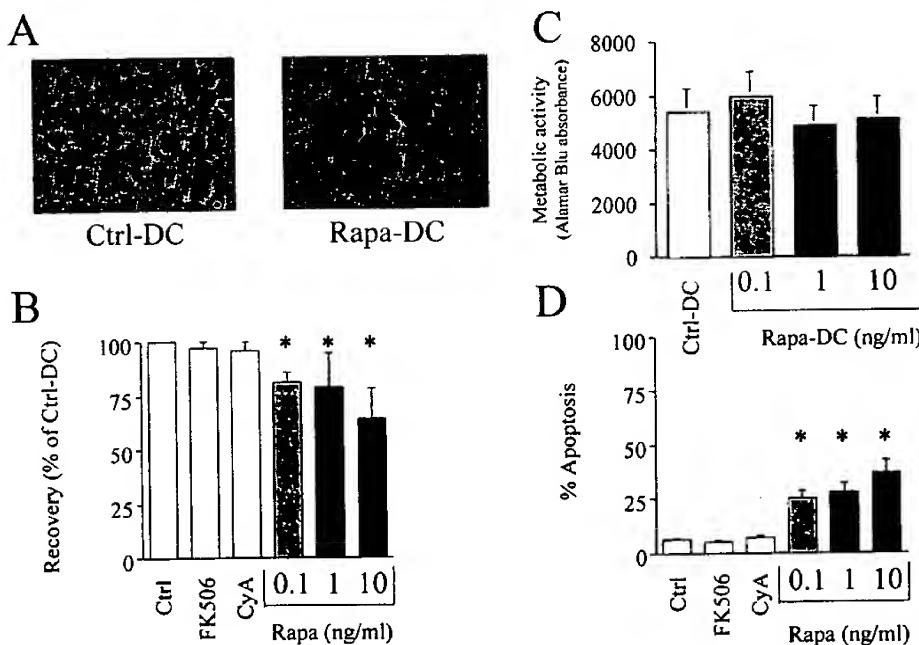


FIGURE 1. Cell recovery and apoptosis of rapa-DC and ctrl-DC. (A) DC were differentiated from monocytes cultured for 7 days in GM-CSF (50 ng/mL) and IL-4 (10 ng/mL) in the absence (Ctrl-DC) or presence (Rapa-DC) of rapamycin. Upon culture with GM-CSF and IL-4, the cells became nonadherent and clustered and had abundant cytoplasm and protruding veils typical of DC. In the presence of rapamycin (right) DC showed a similar morphology. (B) Reduced cell yield after DC development in the presence of rapamycin (0.1–10 ng/mL), FK506 (10 ng/mL), and CsA (500 ng/mL). Data shown are the mean ± SD of 15 experiments; * P <0.05. (C) Metabolic activity of rapa-DC. Metabolic activity was measured by the alamarBlue assay. AlamarBlue is added to cell cultures and incubated at 37°C for 6 to 18 hr. Relative absorbance is read at 530 to 590 wavelengths. Data shown are the mean ± SD of duplicate cultures. The results are representative of two independent experiments. (D) Rapamycin-induced apoptosis in DC. DC were differentiated from monocytes cultured for 7 days in GM-CSF (50 ng/mL) and IL-4 (10 ng/mL) in the absence (Ctrl) or presence of rapamycin (0.1–10 ng/mL), FK506 (10 ng/mL), and CsA (500 ng/mL; CyA). Apoptosis was detected by flow cytometry using annexin V-PI staining. The percentage of apoptosis demonstrates the percentage of annexin V+ PI- cells. Data shown are the mean ± SD of duplicate cultures. The results are representative of five independent experiments. * P <0.05.

TABLE 1. Phenotype analysis of DC differentiated in the presence of rapamycin

	Immature								+ CD40L							
	ctrl-DC		rapa-DC						ctrl-DC		rapa-DC					
			0.1 ng/ml		1 ng/ml		10 ng/ml				0.1 ng/ml		1 ng/ml		10 ng/ml	
Myeloid antigens																
CD14	15	10	10	6	1	4	1	4	nt	nt	nt	nt	nt	nt	nt	nt
Presentation molecules																
CD1a	86	38	93	62*	91	100*	87	81*	88	76	93	86*	92	114*	94	162*
CD1b	70	20	93	41*	90	41*	89	37*	58	30	80	55*	94	95*	95	102*
CD1c	78	22	85	26	89	33*	94	42*	60	16	nt	93	41*	91	39*	
CD1d	5	7	4	6	1	5	1	5	4	5	3	5	3	8	3	11
MHC I	94	78	89	67	87	50*	87	50*	94	205	92	159	92	139*	90	96*
MHC II	94	124	94	122	89	101*	77	66*	91	248	87	235	82	218	69	121*
Costimulatory/signaling molecules																
CD80	10	8	19	10	5	7	6	7	40	16	37	17	13	11	6	4*
CD86	48	28	42	24	15	8*	12	8*	80	112	83	134	87	132	63	60*
CD40	92	34	92	36	90	28*	75	18*	94	111	92	120	93	87*	81	79*
Potential antigen uptake receptors																
CD16	25	14	16	10	4	7*	6	5*	24	13	12	7	6	6*	6	5*
CD32	94	34	nt	nt	nt	42	17*	nt	nt	nt	nt	nt	nt	nt	nt	nt
CD91	27	24	20	22	4	12*	5	10*	nt	nt	nt	nt	nt	nt	nt	nt
CD46	43	30	25	24	18	20*	18	20*	nt	nt	nt	nt	nt	nt	nt	nt
MR	71	43	61	22*	43	15*	44	19*	56	30	52	19*	37	13*	15	9*
Maturation antigen																
CD83	4	6	4	7	4	7	4	6	57	24	61	30	54	28	32	12*

DC were differentiated from monocytes with GM-CSF (50 ng/ml) and IL-4 (10 ng/ml) in the absence (ctrl-DC) or presence of rapamycin (rapa-DC) at different concentrations. Maturation was induced by culture with mJ558CD40L for 24 hr. Results are expressed as percentage and fluorescence intensity. Data were expressed as mean (n=6). * P<0.05 vs. ctrl-DC.

nt, not tested.

differentiated in vitro into fully mature DC by exposure to LPS or CD40L. Rapamycin affected DC maturation. After exposure to CD40L, rapa-DC were less able to up-regulate the molecules involved in antigen presentation (MHC I, MHC II, CD80, CD86, CD40) and CD83 (Table 1). All these effects were dose dependent starting at concentrations of 1 ng/ml. To demonstrate that the effects were not a result of a generic "toxic" effect of rapamycin on differentiating DC but of a specific pharmacologic action, we measured the metabolic activity of rapa-DC by the alamarBlue method. No significant difference was seen before or after maturation in rapa-DC in the concentration range of 0.1 to 10 ng/mL (Fig. 1C).

DC Differentiated in the Presence of Rapamycin Showed Decreased Antigen Uptake Capacity

Immature DC, such as cells derived by culturing monocytes with GM-CSF and IL-4 for 7 days, express a potent ability to uptake soluble external molecules essentially through two mechanisms: a receptor-mediated endocytosis and a fluid phase endocytosis (macropinocytosis). To study the endocytic capacity of rapa-DC, we used two different markers: lucifer yellow (LY), a nonspecific fluid phase marker, and FITC-dextran, which is mainly taken up by the mannose receptor. Immature and mature rapa-DC showed a vigorous inhibition of FITC-dextran uptake (Fig. 2A) and

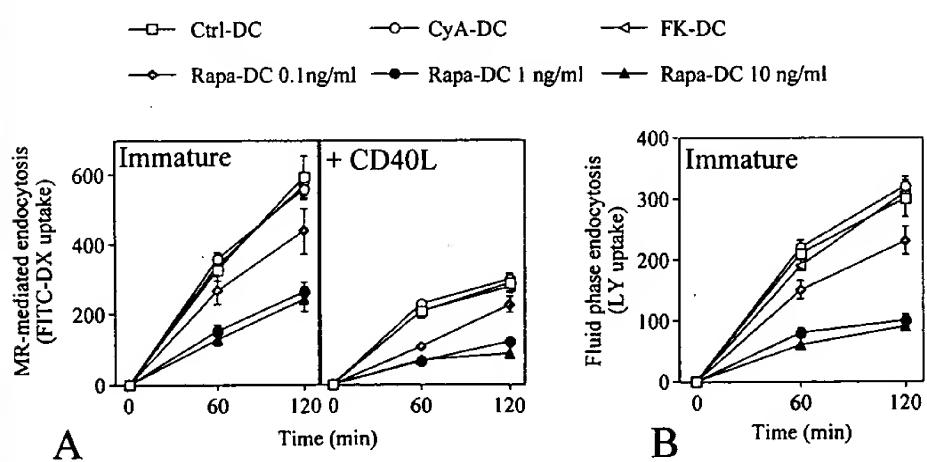


FIGURE 2. Endocytic activity of DC differentiated in the presence of rapamycin. Monocytes were cultured for 7 days with 50 ng/mL GM-CSF, 10 ng/mL IL-4 in the absence (Ctrl-DC) or presence of rapamycin (Rapa-DC), FK506 (10 ng/mL; FK-DC), and CsA (500 ng/mL; CyA-DC). Endocytosis was evaluated as uptake of FITC-dextran (1 mg/mL) (A) or LY (1 mg/mL) (B) and measured by flow cytometry. Results are expressed as mean fluorescence intensity \pm SD (n=8). Statistical differences (P<0.05) were seen starting from 0.1 ng/mL of rapamycin.

immature rapa-DC an inhibition of LY uptake (Fig. 2B). In accordance, rapamycin down-regulated membrane mannose receptor expression in immature and mature cells (Table 1). Immature DC also capture particulate antigens through phagocytosis. To study the phagocytic capacity of rapa-DC, we used four different markers: fluorescent *S. aureus* with or without opsonization, 0.2- μ m fluorescent microspheres, fluorescent dye apoptotic cells, and fluorescent dye dead cells. Ctrl-DC were able to internalize *S. aureus* in a time-dependent manner: 61% and 68% of ctrl-DC phagocytosed *S. aureus*, respectively, after 1 hr and 2 hr of exposure (Fig. 3). This activity was inhibited in a dose-dependent manner by rapamycin: 35% and 47% of rapa-DC (10 ng/mL) phagocytosed *S. aureus* after 1 hr and 2 hr of exposure, respectively. Rapamycin not only inhibited the number of cells able to internalize bacteria but also the intensity of the phagocytic activity as demonstrated by the decrease in fluorescence intensity of rapa-DC positive for *S. aureus* internalization (percentage of inhibition: 23 \pm 6, 66 \pm 10, and 72 \pm 12, respectively, for rapamycin 0.1, 1, and 10 ng/mL) (Fig. 3A). Because phagocytic function may be mediated by antibody opsonization, we also analyzed *S. aureus* internalization by Fc γ receptors. *S. aureus* binding to DC was examined after opsonization with purified rabbit polyclonal IgG antibody. *S. aureus* binding appeared inhibited in rapa-DC (percentage of inhibition: 1 \pm 2, 49 \pm 5, and 72 \pm 6, respectively, for rapamycin 0.1, 1, and 10 ng/mL) (Fig. 3B). As expected, rapamycin also inhibited phagocytosis of opsonized *S. aureus* (Fig. 3A). These findings are consistent with the reduced expression of CD32 and CD16 in rapa-DC. To evaluate the capacity of DC to inter-

nalize dying cells, we tested the phagocytic activity of dead or apoptotic Jurkat cells (Fig. 4A). Internalization of apoptotic or dead cells was inhibited in rapa-DC (Fig. 4A) as was the ability to internalize inert particulate substrates such as latex beads (Fig. 4B), showing a general impaired phagocytic activity of rapa-DC. To evaluate the effects of rapamycin on the already differentiated DC, 10 ng of rapamycin was added to 7-day cultured DC for 48 hr and a maturation signal (10 ng/mL LPS) was added simultaneously or 48 hr later. Under these conditions rapamycin did not inhibit antigen uptake activity (measured as FITC-dextran uptake), did not inhibit mannose receptor expression, and only partially reduced cell recovery (data not shown). Moreover rapamycin did not inhibit the LPS or CD40L induced up-regulation of costimulatory molecules (e.g., CD40, CD80, CD83, and MHC II). These results indicate that rapamycin must be present during differentiation to induce modification of DC activity.

Cytokine Production, T Stimulatory Activity, and T helper 1 Polarization

The ability of rapa-DC to stimulate allogeneic T lymphocytes was examined in MLR. Immature rapa-DC had decreased immunostimulatory capacity compared with immature ctrl-DC. Similarly, the allogeneic T-cell response was inhibited in the presence of LPS or CD40L mature rapa-DC as compared with mature ctrl-DC (Fig. 5A). The proliferating CD4 $^{+}$ T cells were also tested for their capacity to produce IL-4 or IFN-gamma (Fig. 5B). Both IFN-gamma and IL-4 production were reduced in a dose-dependent manner when rapa-DC were used as stimulators. The ability of DC to

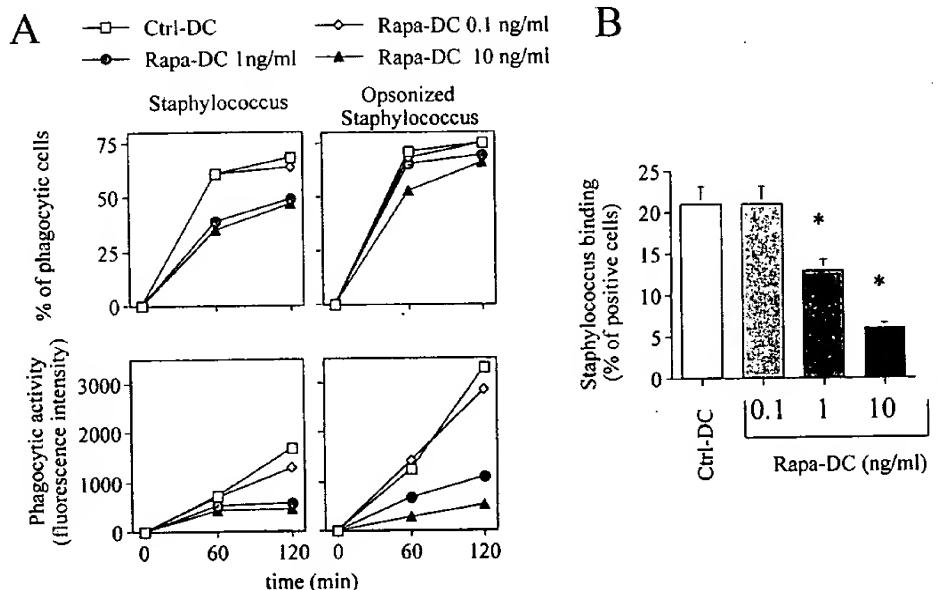


FIGURE 3. Phagocytic activity of DC differentiated in the presence of rapamycin: *S. aureus* internalization. Particle uptake was evaluated by incubating *S. aureus*, either untreated or after opsonization, with ctrl-DC or rapa-DC for 120 min at 37°C or 4°C. After phagocytosis, DC were washed and then incubated in PBS containing EDTA and trypsin for 15 min at 37°C to eliminate the membrane binding. Cells were analyzed by flow cytometry (A). Phagocytic activity was expressed as percentage of positive cells (calculated as positive cells at 37°C – positive cells at 4°C) and mean fluorescence intensity of positive cells (calculated as mean fluorescence intensity of positive cells at 37°C – mean fluorescence intensity of positive cells at 4°C). The results are representative of five independent experiments. Statistical differences ($P < 0.05$) were seen starting from 1 ng/mL of rapamycin. (B) *S. aureus*, either untreated or after opsonization, were incubated with ctrl-DC or rapa-DC for 30 min at 4°C. Binding to DC membrane was evaluated by flow cytometry and expressed as percentage of positive cells \pm SD ($n = 3$). * $P < 0.05$.

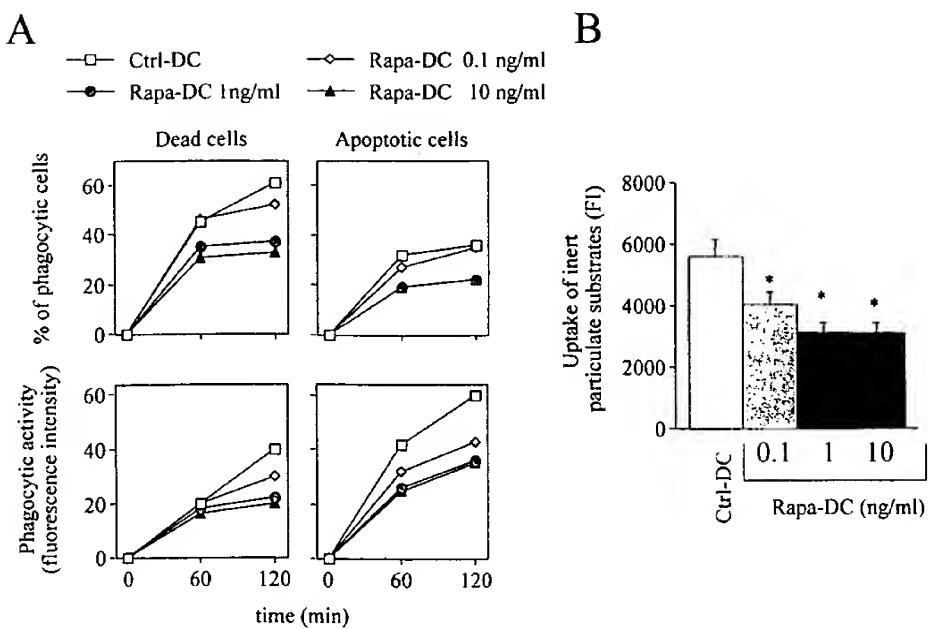


FIGURE 4. Phagocytic activity of DC differentiated in the presence of rapamycin: apoptotic or dead cell internalization. Jurkat cells were labeled with the green fluorescent aliphatic dye PKH2-GL, killed by apoptosis or necrosis, and then coincubated with ctrl-DC or rapa-DC for 120 min at 37°C or 4°C (A). Alternatively FluoSpheres beads were used as a marker of phagocytosis (B). After phagocytosis, DC were washed and then incubated in PBS containing EDTA and trypsin for 15 min at 37°C to eliminate the membrane binding. Washed cells were analyzed by flow cytometry. Phagocytic activity was expressed as percentage of positive cells (calculated as positive cells at 37°C – positive cells at 4°C) and mean fluorescence intensity of positive cells (calculated as mean fluorescence intensity of positive cells at 37°C – mean fluorescence intensity of positive cells at 4°C). The results are representative of five independent experiments. Statistical differences ($P < 0.05$) were seen starting from 1 ng/mL of rapamycin. * $P < 0.05$.

produce IL-12 when stimulated through CD40 or LPS is important for the generation of protective immunity. Stimulation with CD40L resulted in IL-12 production by ctrl-DC, whereas rapa-DC were impaired in their ability to produce both IL-12 and IL-10 (Fig. 5C).

Modifications of Differentiation, Maturation, and Function of DC by Rapamycin Are Not Mediated by Calcineurin Systems But Required Binding to the Cytosolic Receptor Protein FKBP-12

We studied the effect of other immunosuppressive drugs (FK506, CsA, MMF) on DC differentiation from monocytes (Fig. 6). Both calcineurin inhibitors (FK506, CsA) and MMF were unable to modulate DC differentiation and maturation. Moreover, endocytic activity and T-stimulatory activity in MLR were not influenced by these drugs (data not shown). Because FK506 and CsA do not interfere with DC function, we deduced that the calcineurin system is unlikely to play a major role in differentiation, maturation, and activation of human monocyte-derived DC. Because FK506 and rapamycin both bind to FKBP-12, they can act as reciprocal antagonists in a system in which only one drug is active. To determine whether the effects of rapamycin on DC were mediated by FKBP-12, competition experiments with FK506 and CsA were performed. Rapamycin (10 ng/mL) was added during differentiation of DC in the presence of varying concentrations of the calcineurin inhibitors FK506 or CsA. A

10-fold excess of FK506 was able to completely revert rapamycin-mediated inhibition of cell recovery (rapamycin-induced apoptosis) and antigen uptake (Fig. 6B), whereas up to 100-fold excess of CsA showed no effect (data not shown). A similar antagonist effect of FK506 was also seen for phenotype modification and T-cell stimulatory activity inhibition (data not shown).

DISCUSSION

Modulation of T and B lymphocyte function by rapamycin is well documented (4,8). Here, we demonstrated that DC are also a target of rapamycin action. Rapamycin showed complex and unique effects on DC. Unlike other immunosuppressive drugs that modulate DC function (i.e., 1,25(OH)₂D₃ and corticosteroids) (17,18), rapamycin did not block the GM-CSF + IL-4-driven differentiation of monocytes to DC. The expression of CD1a was not inhibited and CD14 expression, which is up-regulated by IL-10, corticosteroid, and 1,25(OH)₂D₃ (17–19), was not increased. Despite having morphologic and phenotypic characteristics of DC, the presence of rapamycin during DC differentiation reduced cell recovery and had an associated increase in apoptosis, findings that are confirmed in a recent report (20), and rapa-DC had a markedly impaired antigen uptake capacity.

Immature DC are very efficient in their antigen uptake capacity (21). They use a variety of mechanisms, which include both receptor-mediated and fluid endocytosis (macropi-

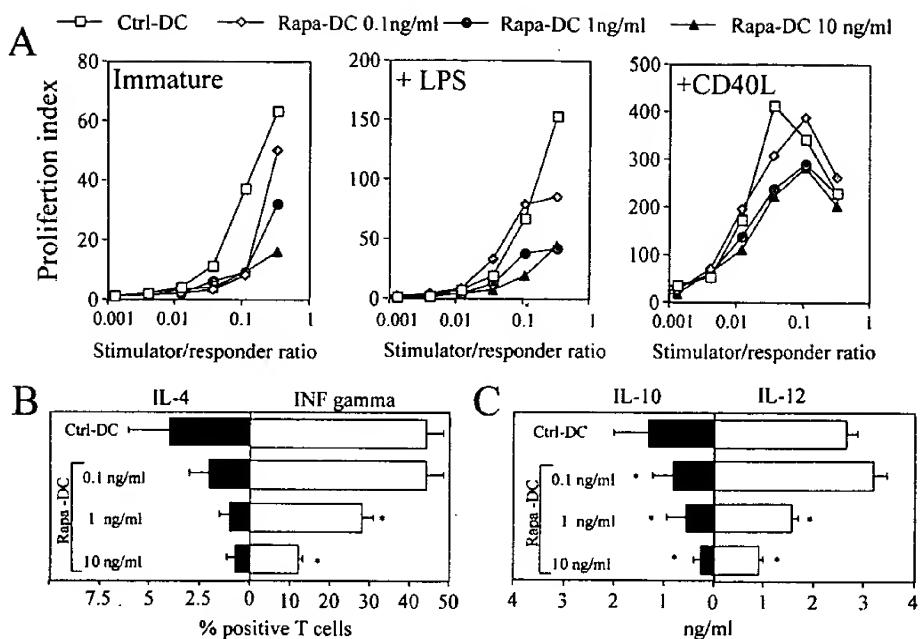


FIGURE 5. Effect of rapamycin on the stimulatory activity in MLR, cytokine secretion, and T-cell polarization. (A) Monocytes were cultured with GM-CSF and IL-4 in the presence (Rapa-DC) or absence (Ctrl-DC) of rapamycin. After 7 days, DC were extensively washed, irradiated (3000 rad), and added in increasing doses to 10⁶/well purified allogeneic responder T cells in 96-well flat-bottomed microtest plates (immature). In a second type of experiment, immature DC were further cultured for 48 hr with LPS (10 ng/mL) or CD40L-transfected cells. Responder cells were allogeneic T cells depleted of autologous APC. Each group was performed in triplicate. Thymidine incorporation was measured on day 5 by a 16-hr pulse with ³H-thymidine (n=6). Statistical differences were seen starting from 1 ng/mL. (B) Production of IFN-gamma and IL-4 was measured by intracellular staining in polyclonal T-cell lines obtained after stimulation of naive cord blood T cells with allogeneic ctrl-DC or rapa-DC after CD40L maturation. Data are expressed as percentage of positive cells. n=6, *P<0.05. (C) Rapamycin affects cytokine production by DC. After 7 days of culture with GM-CSF and IL-4, with (Rapa-DC) or without (Ctrl-DC) rapamycin, cells were washed and stimulated at 10⁶/mL with CD40L (J558LmCD40L). Control groups were DC cultured with untransfected J558L cells. Supernatants were harvested 48 hr later and tested for IL-1 β , IL-10, and IL-12 p70. Results are expressed as mean \pm SD (n=4). *P<0.05.

noctosis) and phagocytosis. Rapamycin inhibited both expression of antigen uptake receptors (mannose receptor, CD16, CD32, CD46, CD91) and receptor-mediated antigen uptake, in addition to fluid endocytosis and phagocytosis. Inhibition was dose dependent and was observed already at the lowest dose tested (0.1 ng/mL). An impairment of antigen uptake capacity is particularly relevant during inflammation of a reperfused graft, in which cell death in the presence of proinflammatory cytokines promotes DC maturation and T-cell priming (indirect presentation) (22). Blocking DC phagocytic activity of dead or apoptotic cells and DC antigen uptake would reduce the likelihood of T-cell priming through indirect presentation and may be a relevant strategy to induce immunosuppression.

Rapamycin also significantly modulated DC CD1 molecule expression. CD1 molecules can be separated into two groups on the basis of sequence homology: group 1, which comprises CD1a, CD1b, CD1c; and group 2, which includes CD1d (23). Rapamycin increased the expression of group 1 molecules. The role of group 1 CD1 molecules in the immune system remains largely unknown (24), and mycobacterium cell wall glycolipids are currently the only known source of microbial antigens that are presented by group 1 CD1 (25). A recent editorial suggested that the recognition of autologous rather than microbial ligands is the primary function of all CD1-

reactive T cells (26)—and not only for group 2 CD1-reactive T cells (NKT cells)—speculating the possibility of a regulatory function also for the group 1 CD1 pathway. If true, then the increased expression seen in the presence of rapamycin should favor the recruitment of regulatory cells.

The expression of other stimulatory and costimulatory molecules found on DC was down-regulated by rapamycin. In the immature state, rapa-DC showed a relatively low expression of costimulatory and HLA molecules. Activation of rapa-DC with CD40L induced CD83 and increased the expression of MHC I, MHC II, and costimulatory molecules (CD80, CD86, CD40) but to a lesser extent than in ctrl-DC. A relatively low expression of costimulatory and HLA molecules on DC generated in the presence of rapamycin was recently reported for human (20) and murine DC (27) and indicates a partial block in DC maturation. This partial block in maturation was also suggested by our studies of cytokine production and T-cell stimulation. Cytokine secretion upon CD40 ligation is a key activity for inducing T-cell priming and polarization. DC are capable of producing IL-10 and IL-12 after maturation. IL-12 plays a relevant role for instructing T cells to differentiate along the T helper 1 pathway (28). The inhibition of IL-12 production after maturation may represent an important immunosuppressive mechanism of rapamycin. Because it was recently reported that DC are able to

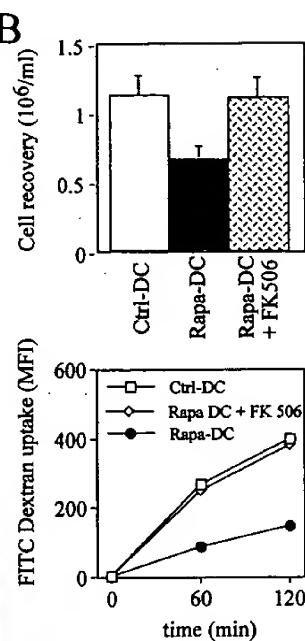
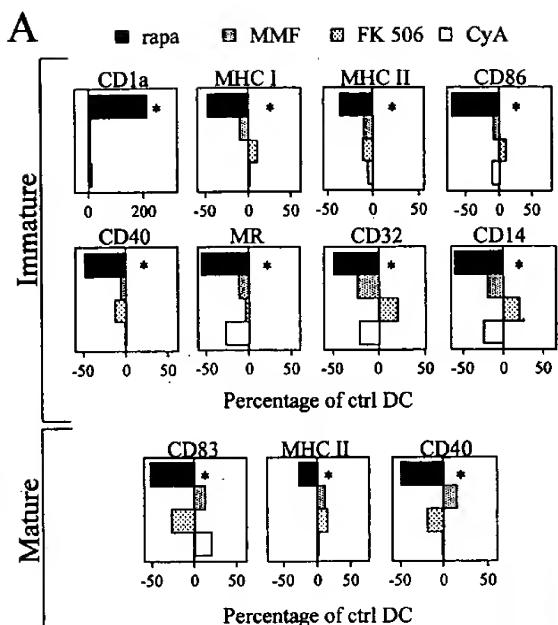


FIGURE 6. Effect of immunosuppressive drug on DC differentiation and maturation. (A) Membrane phenotype analysis of cells cultured for 7 days in GM-CSF (50 ng/mL) plus IL-4 (10 ng/mL) with or without rapamycin (10 ng/mL), tacrolimus (10 ng/mL), CsA (500 ng/mL), and MMF (1 μ g/mL). Cells were labeled with the designed monoclonal antibody and then with FITC-labeled goat anti-mouse Ig. Results are expressed as percentage of variation compared with control, calculated according to the formula (mean fluorescence intensity drug/mean fluorescence intensity control) \times 100. Data shown are mean \pm SD. n = 4, *P < 0.05. (B) Rapamycin-induced effects are blocked by FK506. Cell recovery and FITC-dextran uptake of immature DC differentiated from monocytes after 7 days of culture in the presence or absence of rapamycin (10 ng/mL) and FK506 (100 ng/mL). Data shown are mean \pm SD of duplicate cultures. The results are representative of three independent experiments.

secrete IL-10 instead of IL-12 and to prevent acute allograft rejection (29), we also measured IL-10 secretion by rapa-DC. IL-10 secretion was also decreased, suggesting a general immunosuppressive effect of rapamycin on DC cytokine production rather than modulation to a "tolerogenic" profile.

The effects described in this study were found in a range of rapamycin concentrations from 0.1 to 10 ng/mL, with significant effects observed at 1 ng/mL or less. Importantly, even if rapamycin has been shown *in vitro* to inhibit T-lymphocyte proliferation at a lower concentration (50% inhibition ranging from 0.1 to 30 ng/mL) (8), the concentrations used in our work should be considered clinically highly relevant (30). Whole blood rapamycin concentrations for the 2 mg per day and 5 mg per day doses are reported to be 8.59 ± 0.41 and 17.3 ± 7.4 ng/mL, respectively, for steady state and 12.2 ± 6.2 and 37.4 ± 21 ng/mL, respectively, for peak concentration (31,32). Although rapamycin is extensively bound (approximately 92%) to human plasma proteins (mainly albumin), the mean blood to plasma ratio was 36 ± 17.9 , indicating that rapamycin is extensively portioned into formed blood elements including monocytes. It should also be noted that the concentrations used in our study did not induce a reduction in DC metabolic activity as measured by the alamarBlue assay, indicating that the described effects cannot be attributed to a generic "toxic" effect of rapamycin on differentiating DC but are caused by a specific pharmacologic action.

Regarding the molecular mechanisms by which rapamycin modulates DC function, we have tested and excluded an effect mediated by inhibition of the calcineurin system and an action mediated by inhibition of the IL-2 system (data not shown). Like CsA and tacrolimus, rapamycin binds to cytosolic receptors known as immunophilins (8). CsA binds to the cyclophilin class of immunophilins (cyclophilin A), whereas both FK506 and rapamycin bind to the immunophilins known as FK506 binding proteins (FKBPs), in particular FKBP-12 (8). The fact that both calcineurin inhibitors FK506

and CsA do not modify DC function indicates that the calcineurin system does not play a relevant role in DC differentiation and function. However, although the calcineurin system did not seem relevant for rapamycin action, FK506 was completely antagonistic to the inhibitory action of rapamycin on DC. This observation indicates that binding to FKBPs plays a role in the rapamycin-mediated effects. Rapamycin is structurally related to FK506, but only the rapamycin-FKBP complex binds and inhibits the function of the serine-threonine kinase mammalian target of rapamycin (TOR) (8), which is involved in protein synthesis and cell cycle progression and may be the way by which rapamycin modulates DC function. The precise mechanism by which rapamycin inhibits DC antigen uptake remains to be determined. In this context, it is important to note that the Rho GTPases CDC42 and Rac, which interfere with the endocytic activity of DC (33,34), complex with and activate the p70 S6 kinase (35) that belongs to the central signaling pathway disrupted by rapamycin (8). In addition, rapamycin's inhibition of TOR signaling down-regulates protein translation and has been demonstrated to suppress actin synthesis (36).

Typical treatments for the prevention of rejection include the administration of steroids, azathioprine or MMF and CsA or FK506 and anti T-cell globulin or anti-CD3 antibody. With the exception of steroids, as previously described, rapamycin seemed to be the immunosuppressive drug that was uniquely able to influence DC function. Rapamycin had several effects on differentiating DC, including inhibition of antigen uptake, a partial block of maturation, a decrease in cytokine secretion, and a reduced capacity in inducing T-cell responses. Although the exact molecular mechanism of these rapamycin-induced effects and their importance *in vivo* must be elucidated, suppression of DC may contribute to the potent actions of rapamycin in the prevention of allograft rejection and other immune-mediated phenomena.

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1. Transplantation:
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thanks!

Dendritic Cells, Interleukin 12, and CD4⁺ Lymphocytes in the Initiation of Class I-restricted Reactivity to a Tumor/Self Peptide

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ABSTRACT: Cell-mediated immunity involving CD8⁺ lymphocytes is effective in mediating rejection of murine mastocytoma cells bearing P815AB, a tumor-associated and self antigen showing similarity to tumor-specific shared antigens in humans. Although this antigen may act as an efficient target for class I-restricted responses in immunized mice, neither P815AB expressed on tumor cells nor a related synthetic nonapeptide will activate unprimed CD8⁺ cells for *in vivo* reactivity, measured by skin test assay. We review evidence showing that the failure of P815AB to initiate CD8⁺ cell reactivity may be due to defective recruitment of accessory and Th1-like cells to the afferent phase of the response initiated by transfer of mice with dendritic cells pulsed *in vitro* with the P815AB peptide. Although the co-presence of a T helper peptide in dendritic cell priming *in vitro* with P815AB may compensate for the poor generation of accessory and Th1 cells in the adoptively transferred mice, recombinant IL-12 can replace the helper peptide in both effects. Effective priming to P815AB *in vivo* is achieved by either exposing dendritic cells to IL-12 prior to P815AB priming or administering the recombinant cytokine *in vivo*. Different approaches suggest that IL-12 may act both on accessory cells to improve presentation of previously undescribed class II-restricted epitopes of P815AB and on CD4⁺ cells to improve recognition of such epitopes. In particular, at the CD4⁺ cell level, IL-12 apparently acts as an adjuvant and an inhibitor of anergy induction. These data offer useful information for developing vaccination strategies using dendritic cells and class I-restricted tumor peptides in humans.

KEY WORDS: tumor peptides, dendritic cells, IL-12, anergy.

I. INTRODUCTION

The specificity of the immune response is orchestrated by two arms of the immune system, namely B and T cells, each possessing vast arrays of clonally distributed antigen receptors. It is the fundamental diversity of T and B cell receptors that endows the system with the ability to recognize foreign antigens and to discriminate self from nonself. For the generation of an effective antitumor response, two requirements must be met. First, the tumor must present antigens that can be

recognized by T cells and are not found on most normal cells. Second, the immune system must distinguish these antigens as distinct from self. Much evidence now exists that tumors may possess specific antigens recognizable by T cells.¹ Therefore the goal of antitumor immunotherapy is to initiate reactivity, or break tolerance, to these antigens while preserving self tolerance. Recently, novel approaches were developed in animal model systems that may allow for the onset of strong reactivity to otherwise poorly immunogenic epitopes of malignant cells. On the one hand,

** Abbreviations used in this paper: DC, dendritic cells; DTH, delayed-type hypersensitivity; tt, tetanus toxin (peptide); FWI, increase in footpad weight.

dendritic cells (DC) have been found to be unmatched in their ability to present peptide antigens to T cells, thus initiating immune reactivity.² On the other, synthetic peptides corresponding to identified tumor antigens are available that can be used to selectively trigger protective antitumor responses.^{3,4} Finally, recombinant cytokines can be used in vaccination procedures to potentiate or permit the onset of a sufficient and/or appropriate immune response to tumor antigens.⁵

It is also clear that at least two different pathways exist for the processing of antigens recognized by T cells. CD4⁺ T helper cells tend to recognize exogenously derived antigens that are endocytosed by antigen-presenting cells (APC) and degraded in endosomal compartments. Peptide fragments of the antigen will then associate with class II MHC molecules, and this complex is displayed on the APC surface. CD8⁺ cells, including classic cytolytic T lymphocytes (CTL), recognize peptides derived from endogenously synthesized proteins. One major unanswered question in antitumor immunity is whether the failure of the immune system to reject poorly immunogenic tumors is due to the absence of tumor-specific CTL or to a failure to activate T helper (Th) cells necessary to support CTL priming.⁶ In addition, because of the fundamental functional dichotomy of CD4⁺ cell responses in experimental models of infection,⁷ it is possible that the failure of Th cells to support CTL priming may be due not only to the poor activation of the appropriate CD4⁺ subset, but also to the activation of an inappropriate CD4⁺ subset with inhibitory activity on the development of cell-mediated (or Th1-dependent) reactivity. The latter possibility would be expected to involve production of inhibitory (Th2) cytokines, such as interleukin (IL)-4 and IL-10, that antagonize the onset/expression of cell-mediated immunity,⁸ which is instead favored by Th1 or type-1 cytokines, such as interferon- γ (IFN- γ) and IL-12.⁹

Our group has been developing approaches to determine whether the failure of immunocompetent mice to reject poorly immunogenic tumors is due to poor CTL recognition of relevant peptide epitopes or to a failure to generate adequate or sufficient Th cell responses. In addition, we investigated the efficacy of vaccination procedures combining the use of peptide-pulsed DC and recombinant cytokines. Finally, we attempted to define

experimental conditions suitable for the selective triggering of antitumor but not autoimmune responses when the synthetic peptide, related to a major tumor rejection antigen, is also expressed by a minority of normal cells in the host.

II. CLASS I-RESTRICTED DELAYED-TYPE HYPERSENSITIVITY AS A MEANS OF MONITORING CD8⁺ CELL ACTIVATION *IN VIVO* TO TUMOR PEPTIDES

CTL are a critical component of host immunity to tumors. As mentioned above, CTL typically recognize peptides derived from endogenously synthesized proteins and presented by MHC class I molecules on the surface of target cells. Peptides recognized by tumor-specific CTL are being defined for several human tumors and MHC class I alleles. Although the recognition of peptide class I complexes is sufficient to trigger target cell lysis, priming of CTL responses requires the presentation of the relevant antigen by professional APC, among which DC appear to be efficient APC for CTL induction, thus representing an attractive adjuvant for immunizations using tumor peptide antigens.¹⁰ Whereas assaying of CTL activity *in vitro* could be an important correlate of the *in vivo* effects of immunization, the availability of peptide-based skin tests, analogous to the conventional delayed-type hypersensitivity (DTH), might be instrumental in optimizing immunization strategies and documenting an *in vivo* response.

DTH is the standard *in vivo* assay for T cell-mediated immunity when the antigen is given in the form of a protein. In humans and mice, DTH responses are principally due to CD4⁺ cells, whose activation requires that antigen be processed and presented by class II-expressing APC. Because soluble proteins in the extracellular fluids are excluded from the class I-restricted pathway of antigen presentation in most cells, CD8⁺ lymphocytes are not, in general, activated by cutaneous challenge with protein antigens, which results in lack of skin tests to assess CD8⁺ T cell responses in humans.

By using well-characterized, class I-restricted synthetic peptides related to rejection antigens

of immunogenic (tum⁺) variants of mouse mastocytoma P815,¹¹ we reported on the suitability of a mouse skin test to detect CD8⁺ cell responses *in vivo* following transfer of tumor peptide-pulsed DC. In particular, we observed DTH reactions in immunized mice challenged subcutaneously with class I-binding peptides related to rejection antigens of mutagenized P815 cells.¹² As observed by others by skin test in virally infected mice challenged with viral peptides,¹³ the intrafootpad injection of tum⁺ peptides resulted in a dose-dependent DTH that peaked at approximately 24 h. The response was mediated by CD8⁺ cells and could be induced by previous vaccination of mice with live tumor cells or adoptive transfer with peptide-pulsed DC. These sensitization procedures resulted in an immunologically specific footpad reaction detectable for up to six months after priming.

Because the assay system for measuring the class I-restricted DTH response to tumor peptides is extensively used in the experimentation reviewed in the present article, it may be appropriate to briefly summarize the experimental conditions of the assay. Approximately 50 µg of peptide solubilized in 30 µl of vehicle are inoculated into the left hind footpads of mice adoptively transferred intravenously with peptide-pulsed DC two weeks earlier. The right hind footpad receives the same volume of vehicle with or without an irrelevant peptide. The DTH reaction is recorded 24 h later, when the animals are killed, their hind feet cut off at the hair line, and weights recorded as a measure of swelling, edema, and cellular infiltration. Results are expressed as the increase in footpad weight (FWI) over that of the vehicle (or irrelevant peptide)-injected counterpart, which serves as an internal control for each individual mouse.

The preliminary results we obtained by the use of strongly immunogenic tum⁺ peptides of P815 cells indicated the occurrence of strong antigen-specific footpad reactivity in mice primed with peptide-pulsed DC.¹² In contrast, no response was observed in mice receiving an intravenous injection of free peptide or unpulsed DC. Reactivity was selectively abrogated by CD8⁺ cell depletion (Figure 1). In subsequent studies, we addressed the issue of whether CD4⁺ cells would be required for the induction of the class I-restricted DTH. We also extended the study to a

tumor peptide related to a major rejection antigen of P815 cells, which is effectively recognized by CTL from preimmunized mice but is incapable of triggering a primary CTL response *in vivo* in the absence of adjuvants.

III. GENE P1A, TUMOR REJECTION ANTIGEN P815AB, AND THE CORRESPONDING SYNTHETIC PEPTIDE

Gene P1A, which is silent in normal mouse tissues except testis and placenta, but is expressed in mastocytoma cells, encodes a protein that includes a nonapeptide representing a tumor rejection antigen (P815AB) that comprises two distinct T cell epitopes.¹⁴⁻¹⁶ This antigen was defined by CTL clones derived from spleen cells of DBA/2 mice that had rejected immunogenic (tum⁺) vari-

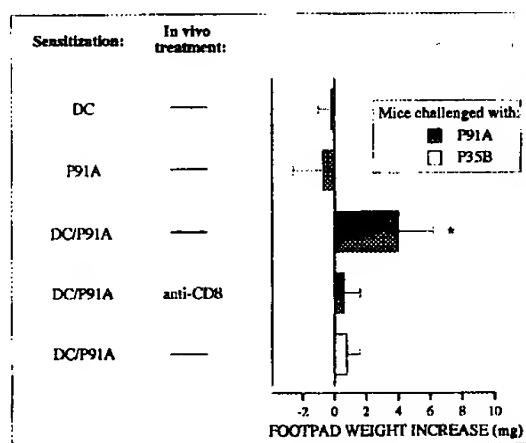


FIGURE 1. Ability of peptide-pulsed DC to induce class I-restricted DTH. DBA/2 mice received an intravenous injection of DC pulsed *in vitro* with the highly immunogenic tum⁺ peptide P91A (DC/P91A; 5 µM for 2 h) two weeks before assessment of their footpad response to challenge with P91A or the antigenically unrelated P35B tum⁺ peptide. A group of mice was also treated with anti-CD8 (2.43) mAb 24 h before the intrafootpad challenge. Control mice were injected i.v. with 5 µg of P91A in the absence of DC (P91A) or with unpulsed syngeneic cells (DC). Values are the mean difference (\pm SD) in weight between experimental (left) and saline injected (right) footpads. *, $p < 0.05$ (peptide challenge vs. saline). In all experiments involving the use of DC, spleen cells were used as a source of DC-enriched fractions, which were purified in 1% normal mouse serum, yielding a population of $> 90\%$ N418⁺ cells.

ants of P815 cells. The observation that P815A and B are lost by tumor cells that escape tumor rejection *in vivo* provided a rigorous demonstration that these antigens have a significant role in the antitumor response occurring *in vivo*.¹⁷ However, the same studies demonstrated that although constituting a potential target for rejection responses, P815AB may not be sufficiently immunogenic on its own.¹⁷

In line with those previous studies, we found that a synthetic nonapeptide related to P815AB fails to elicit class I-restricted DTH after sensitization of DBA/2 mice with irradiated tumor cells or adoptive transfer of P815AB-pulsed DC.¹⁸ However, unresponsiveness can be overcome by immunization with DC pulsed *in vitro* with a physical mixture of P815AB and tum⁺ peptides. Also highly effective in triggering P815AB-specific DTH is the combined use of P815AB and class II-restricted peptides of tetanus toxin (tt) or *Plasmodium berghei* circumsporozoite protein.¹⁸ One major finding in these studies was that ablation of CD4⁺ cells would negate induction of reactivity when it occurred at the time of host sensitization with double (P815AB + tt)-pulsed DC (Figure 2). This led us to hypothesize that defective activation of CD4⁺ cells might contribute to the poor immunogenicity of P815AB. As a matter of fact, in DTH reactions to tum⁺ peptides, their intrafootpad administration in saline (which results in presumably short half-lives) may preclude recruitment of CD4⁺ memory cells to the effector phase of the response. This would explain why reactivity is class I-restricted and is unaffected by late ablation of CD4⁺ cells. However, priming of CD4⁺ cells might be possible or even necessary during the induction phase, provided that the peptides contain immunogenic T helper epitopes, and in fact ablation of CD4⁺ cells at the time of DC transfer blocks induction of DTH reactivity. If CD4⁺ cells are required for induction of class I-restricted DTH to tumor peptides, poor ability of P815AB to bind class II molecules might contribute to the defective activation of the T cell response, including initiation of DTH reactivity. According to this view, the adjuvant effects of tum⁺ or helper peptides, used in combination with P815AB for DC pulsing, might rely on the ability of the former peptides to provide class II-binding epitopes and to result in

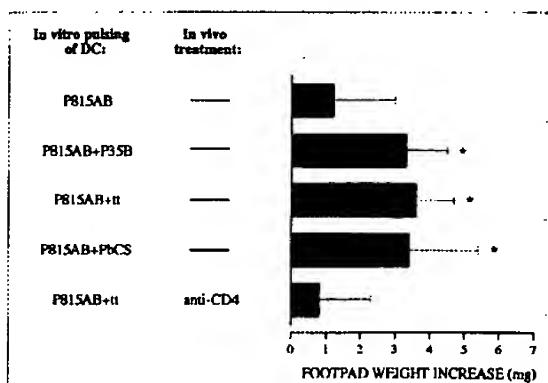


FIGURE 2. Ability of tum⁺ (P35B) or T helper peptides (tetanus toxin: tt947-967; *Plasmodium berghei*; PbCS20-39) to induce P815AB-specific DTH when used in combination with P815AB for DC pulsing. DC exposed to P815AB + P35B, P815AB + tt, or P815AB + PbCS were transferred into mice either untreated or treated concurrently with anti-CD4 (GK1.5) mAb. The DTH was recorded two weeks after sensitization. *, $p < 0.05-0.01$ (peptide challenge vs. saline).

CD4⁺ cell activation. Cytokines released by CD4⁺ cells in close proximity to CTL precursors would permit effective CTL priming. However, two major questions remain unanswered: (a) by which mechanisms the copresence of helper epitopes on DC permits effective presentation/recognition of P815AB CTL epitopes? and (b) by which mechanisms will the activated CD4⁺ cells support CD8⁺ cell priming to P815AB? In regard to the first question, it is interesting to note that DC can produce IL-12,¹⁹ a critical cytokine for the initiation of cell-mediated immunity.²⁰ A subsequent series of experiments was therefore devoted to clarify whether defective production of IL-12 by DC pulsed with P815AB alone might be responsible for the defective presentation and/or recognition of class I-restricted epitopes of P815AB.

IV. REQUIREMENT FOR HELPER PEPTIDES OR RECOMBINANT IL-12 IN THE INITIATION OF CLASS I-RESTRICTED REACTIVITY TO P815AB *IN VIVO*

IL-12 induces a number of biologic effects that are consistent with its potential role as an

antitumor agent, including activation of IFN- γ releasing NK cells, stimulation of Th1 reactivity, enhancement of CTL responses, and inhibition of angiogenesis. Each of these biologic properties could mediate or contribute to the antitumor effects observed in mice bearing a variety of malignancies and treated locally or systemically with IL-12.²¹ Although much evidence points to a critical role for IL-12 as an adjuvant for vaccines in infectious diseases,^{22,23} little is known about such potential applications in cancer.²⁴ In a recent study, combined treatment of Meth A tumor-bearing mice with a nonameric peptide (related to mutated p53) and IL-12 resulted in tumor regression, and this correlated with enhanced induction of peptide-specific CTL.²⁵ In mice cured of their tumors by IL-12 treatment, the majority of work indicates that T cells are critical in mediating the antitumor effects of the exogenous cytokine, but the relative contributions of different T cell subsets may depend on the experimental conditions. In addition to its therapeutic effects, IL-12 has been suggested to play a role in the spontaneous rejection of a rat tumor that induces IL-2 production *in vivo*.²⁶

APC, including DC, can produce IL-12 and drive the development of IFN- γ -producing Th1 cells from naive CD4 $^{+}$ cells *in vitro*, provided that priming occurs in the absence of IL-4, which strongly inhibits Th1 development.¹⁹ Therefore, whether Th1 cells responsible for cell-mediated immunity develop in response to antigen may depend on the nature of the antigenic stimulus, which may or may not be able to induce IL-12 by APC, as well as on the possible release of IL-4 by different cell types. The latter include a subpopulation of memory CD4 $^{+}$ cells that has been shown to secrete IL-4 after primary stimulation *in vitro*,²⁷ as well as naive T cells responding to low intensity signaling of their receptors, such as that mediated by low affinity peptides.²⁸

Based on the previous finding that induction of DTH to P815AB requires the co-presence of a helper peptide and a functional CD4 $^{+}$ cell compartment,²⁹ we investigated the possible role of endogenous IL-12 in the adjuvant effect of helper peptides, such as tt. We found that the adjuvant effect of tt was associated with the appearance of early and late IL-12 transcripts in the spleens

of mice receiving double (P815AB + tt)-pulsed DC. This effect also correlated with a late IFN- γ response by CD8 $^{+}$ cells, and was negated by serologic ablation of endogenous IL-12 at the time of cell transfer. Most importantly, exogenous IL-12 administered *in vivo* to the DC recipient mice, or used *in vitro* for activation of DC prior to peptide pulsing, could substitute for the T helper peptide in initiating DTH following transfer of DC pulsed with P815AB alone (Figure 3 and Reference 29). Additional studies in this model suggested the following: (a) exogenous IL-12 requires both CD4 $^{+}$ and CD8 $^{+}$ cells for inducing P815AB specific DTH; (b) the immune response initiated by IL-12 relies on later production of IL-12 by the host; (c) the early adjuvanticity of the exogenous IL-12 involves improved presentation and/or recognition of class II-restricted epitopes of P815AB; (d) the adjuvant activity of IL-12, resulting in the onset of

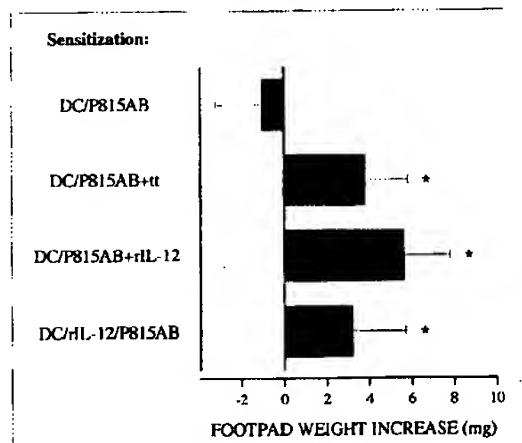


FIGURE 3. Ability of recombinant IL-12 (rIL-12) to replace the helper peptide in initiating footpad reactivity to challenge with P815AB. Mice were transferred with DC pulsed with P815AB alone (DC/P815AB, control) or with a combination of P815AB and tt (DC/P815AB + tt). Alternatively, mice received single (P815AB)-pulsed DC and were treated *in vivo* with 100 ng rIL-12 for 7 d commencing on the day of DC transfer (DC/P815AB + rIL-12). A group of mice was instead treated with DC exposed to rIL-12 *in vitro* overnight (100 ng/ml), during the purification procedure (prior to extensive washing and pulsing with P815AB alone; DC/rIL-12/P815AB). The DTH reaction was measured two weeks after DC transfer. *, $p < 0.01-0.001$ (peptide challenge vs. saline).

an anti-P815AB response when used in combination with DC transfer, could be detected not only in terms of antigen-specific DTH, but also in terms of ability to inhibit local growth of P815 cells and revert the outcome of an otherwise lethal challenge with the tumor (Figure 4).

A number of points could be made from these experiments. First, P815AB may possess class II-restricted epitopes recognizable by specific CD4⁺ cells from suitable immunized mice. This would explain the high IFN- γ production by CD4⁺ cells observed *in vitro* on culturing the latter cells from suitably immunized mice with P815AB in the presence of APC.²⁹ Second, in the face of P815AB failure to initiate Th1 differentiation after transfer of peptide-pulsed DC,^{18,29} the helper peptides (including tt) may act to compensate for the defective recognition of class II-restricted epitopes of P815AB in a primary response. Considering that no IL-4 is induced by P815AB *in vitro*,²⁹ the Th1

response could be intrinsically insufficient in the absence of helper peptides. Third, the adjuvant effect of the helper peptide could be assigned to its ability to induce IL-12 by DC. Taken together, these data supported a primary role for endogenous/exogenous IL-12 and CD4⁺ cells in initiating class I-restricted reactivity to P815AB.

The traditional view of differentiation of CD8⁺ cells relies on participation of CD4⁺ cells to provide help in the form of cytokines thought to be necessary for proliferation and activation of functional CTL properties. Although the exact requirements *in vivo* for costimulatory molecules and cytokines are not entirely known,³⁰ IL-12 favors the emergence of a Th1 cytokine profile during the differentiation of CD4⁺ cells,¹⁹ and is also critical for the activation of differentiated T cells of both CD4⁺ and CD8⁺ phenotype.³¹ Recent studies in human and murine systems suggest a central role for CD4⁺ cells in initiating, effecting, and maintaining antitumor immunity.³² Together with the characterization of tumor-associated antigens recognized by CD8⁺ CTL, a more thorough understanding of CD4⁺ cell-directed immunoregulation may be critical to the success of peptide-based vaccination strategies. Undoubtedly, the development of broadly applicable and effective therapies targeting MHC class II-restricted tumor epitopes will require the identification of these antigens. Our studies with P815AB indicate that the tumor peptide does possess a class II-restricted epitope, yet under conditions of no added adjuvanticity, such as that provided by the use of helper peptides or exogenous IL-12, this epitope might not be adequately presented by DC or recognized by CD4⁺ cells. Therefore, our subsequent experiments were aimed at identifying possible helper epitopes in P815AB that could be presented and/or recognized effectively in the presence of helper peptide-induced, or externally added, IL-12.

Although P815AB may possess class II-binding ability, either a low binding affinity or the nature of a self peptide might prevent effective recognition by CD4⁺ cells in a naive host. In line with this possibility, our most recent studies with P815AB have been focusing not only on the possible identification of helper epitopes but also on the mechanisms governing activation vs. anergy in CD4⁺ cells with specificity for this tumor-associated and self antigen peptide.

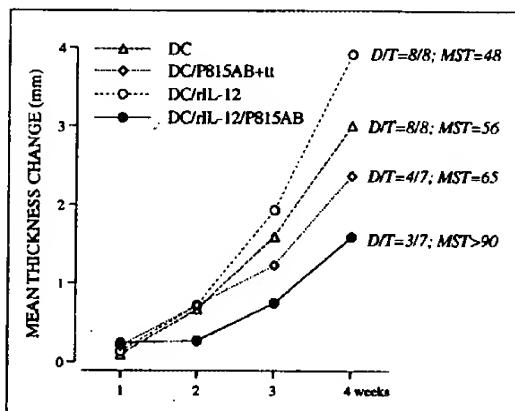


FIGURE 4. Effect of host transfer with IL-12-treated and P815AB-pulsed DC on intrafootpad challenge with P815 tumor cells. DC were exposed sequentially to IL-12 and P815AB (DC/IL-12/P815AB). Two weeks after cell transfer, mice received a footpad challenge with 10^6 tumorigenic P815 cells, and were monitored for local growth of the tumor and mortality parameters. Additional groups of mice received either unpulsed DC, treated (DC/IL-12) or not (DC) with IL-12, or DC pulsed with a combination of P815AB and tt but no IL-12 (DC/P815AB + tt). Tumor growth in experimental footpads was measured at different weeks post-challenge by means of a digital micrometer (mean thickness change relative to control counterparts). D/T, number of dead mice over total animals injected; MST, median survival time (days).

V. THE ADJUVANT EFFECT OF IL-12 MAY INVOLVE INCREASED PRESENTATION AND/OR RECOGNITION OF CLASS II-RESTRICTED EPITOPE OF P815AB

The studies summarized so far indicated that the adjuvant effect of exogenous IL-12 might occur through involvement of class II-restricted epitopes of P815AB.²⁹ Two approaches were employed in subsequent studies to demonstrate the occurrence of such epitopes in the tumor peptide. The first involved assaying P815AB for the ability to inhibit antigen presentation using a class II-restricted OVA-specific T cell hybridoma. In competition experiments using Th peptides as positive controls, we found that P815AB was as effective as the Th peptides in inhibiting presentation of the OVA peptide to class II-restricted hybridoma cells. Yet, on assaying the persistence of MHC-P815AB complexes, we found that these complexes were apparently characterized by poor stability when compared with the persistence of MHC-Th peptide complexes (Figure 5). This might result in inadequate activation of the APC function in DC. As a second approach to the identification of Th epitopes in P815AB, we generated CD4⁺ cell clones with specificity for the tumor peptide, using spleens of mice immunized with P815AB and IL-12. The overall conclusions of these studies³³ can be summarized as (a) the inadequate activation of P815AB-specific CD4⁺ cells observed *in vivo* occurs in the face of class II-restricted epitopes that are, instead, readily detected by functional assays *in vitro*, including antigen-specific production of IFN- γ by *in vivo*-primed CD4⁺ cells and clones, CD4⁺ cell-dependent generation *in vitro* of a primary IFN- γ response driven by externally added IL-12, and the ability of P815AB to inhibit the simultaneous presentation of an antigen peptide to a class II-restricted T cell hybridoma; (b) the nature of a self peptide might contribute to the low immunogenicity of P815AB in a naive host via induction of T cell unresponsiveness; (c) should reactivity to P815AB be regulated in a naive host by mechanisms governing reactivity to self peptides, exposure of mice to P815AB in the absence of adjuvants would be expected to induce deletional and/or reversible

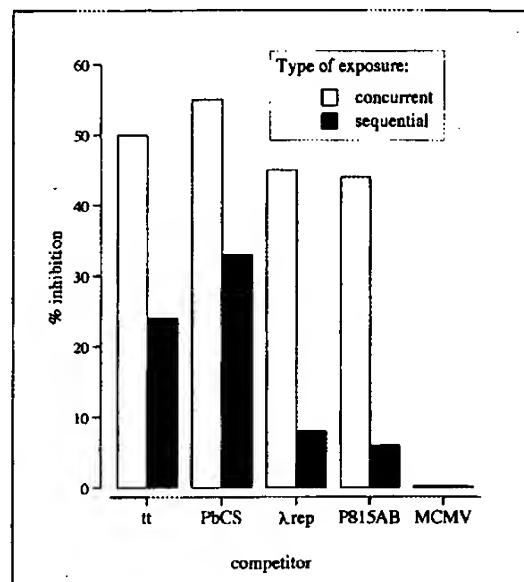


FIGURE 5. Persistence of MHC-P815AB complexes as measured in concurrent vs. sequential exposure in competition experiments for antigen presentation. P815AB was examined for ability to inhibit antigen presentation using the class II-restricted OVA-(323-339)-specific T hybridoma DO11.10. Also used as reference competitor peptides were high affinity tt and PbCS peptides (see legend to Figure 2), intermediate affinity λ-repressor (λ rep) peptide, and a murine cytomegalovirus (MCMV) peptide known to lack class II-restricted epitopes. APC were pulsed with a combination of competitor and OVA peptides (concurrent exposure) or were first exposed to the competitor for 4 h (sequential exposure), washed, and incubated with OVA and DO11.10 cells for 24 h. Supernatants were added to indicator HT-2 cells for assessment of IL-2-dependent growth. Results are expressed as the % inhibition of radiolabel uptake by HT-2 in the presence of a control supernatant (i. e., generated without competitor peptide).

effects in the responding T cells, including heightened T cell receptor (TCR) threshold for IL-2 production³⁴ and negative regulatory functions.³⁵

VI. IL-12 ACTS AS BOTH AN ADJUVANT AND AN INHIBITOR OF ANERGY INDUCTION TO P815AB *IN VIVO*

As an approach to the study of the mechanisms of P815AB unresponsiveness *in vivo*, we investigated whether a first exposure (in a naive host) to

P815AB-pulsed DC in the absence of adjuvants would affect the subsequent development of functional CD8⁺ cells. We assayed the ability of the tumor peptide alone to affect the induction of DTH when administered two weeks before optimal sensitization with the peptide (P815AB + tt) combination.³³ We found that the ability of the peptide combination to initiate P815AB-specific T cell reactivity was lost in mice previously exposed to P815AB. In addition, we observed the following: (a) the antigen-specific unresponsiveness associated with exposure to P815AB alone was a durable, but reversible, phenomenon: failure to respond to an effective priming was, in fact, maximal at two weeks, but no longer detectable at two months; (b) when compared with lymphocytes from optimally immunized mice, CD4⁺ cells from P815AB (alone)-exposed animals failed to produce IL-2 *in vitro*, and their CD8⁺ cells did not provide a late IFN- γ response; (c) under *in vitro* conditions, no significant increase in T cell apoptosis was observed in the unresponsive mice relative to lymphocytes from optimally immunized animals. Therefore, antigen-specific anergy, but not apoptosis, appeared to be induced by an ineffective transfer with P815AB-pulsed DC.

Considering that serologic ablation of endogenous IL-12 at the time of transfer with double-pulsed DC blocks the induction of P815AB-specific DTH and that recombinant IL-12 can substitute for the helper peptide,^{29,33} the following scenario could be envisioned: (a) the necessity and sufficiency of IL-12 in the model system involving double-pulsed DC could relate not only to an intrinsic deficiency of the developing Th1-like response, but also to regulation of a balance between activation and tolerogenic signals acting on T cells; (b) CD4⁺ cells may be a primed target, whether direct or indirect, for both types of IL-12 activity; and (c) the production of IL-2 by CD4⁺ cells may be a critical factor in permitting functional reactivity to P815AB.

VII. IL-12 CAN BOTH PREVENT AND REVERT ANERGY TO P815AB

Endogenous IL-12 may have a role in the induction of Th1-mediated autoimmune diseases,^{24,36} and exogenous IL-12 exacerbates au-

toimmunity in experimental models.^{37,38} Because anergy to P815AB could not be overcome by subsequent coimmunization with a Th peptide, we wondered whether the use of recombinant IL-12 would restore responsiveness to the tumor peptide. A tolerogenic priming was followed at two weeks by transfer with DC exposed sequentially to IL-12 and P815AB *in vitro*, and the recipient mice were treated with IL-12 for 2 d. We found that this immunization procedure was highly effective in reverting anergy into responsiveness, as shown by assessment of DTH reactivity to P815AB and antigen-specific production of IL-2 by CD4⁺ cells and of IFN- γ by CD8⁺ cells *in vitro*. IL-2 production, in particular, was dramatically enhanced by IL-12 treatment.³³

A subsequent series of experiments was designed to investigate whether unresponsiveness to P815AB could also be induced by superantigen-induced tolerance resulting from pre-exposure of mice to staphylococcal enterotoxin B (SEB). SEB, in fact, is a bacterial superantigen that specifically activates T cells bearing V β 8 TCR domains, which eventually leads to a long-lasting state of clonal anergy accompanied by selective cell death in the targeted CD4⁺ subset.^{39,40} In addition, we investigated the possible effects of serological ablation of endogenous IL-12 occurring at the time of an otherwise effective priming with double (P815AB + tt)-pulsed DC. Finally, we studied the possible reversal of these effects by the use of recombinant IL-12 (Figure 6). We found that a previous exposure (on day -28) of mice either to SEB or to double-pulsed DC in concurrence with IL-12 neutralization was as effective as a tolerogenic priming with P815AB (alone)-pulsed DC in blocking the induction phase of reactivity (by transfer with double-pulsed DC, on day -14 relative to DTH assessment). However, the effects of each of these tolerogenic procedures (i. e., SEB, double-pulsed DC + anti-IL-12, or single-pulsed DC) were reversed by the use (on day -14) of DC pre-exposed *in vitro* to IL-12 prior to P815AB pulsing.

The mechanisms of action of IL-12 in breaking tolerance to P815AB are unclear at present, but could involve improved costimulus competence of APC to overcome the heightened TCR threshold of anergic CD4⁺ cells,⁴¹ restore IL-2 production,⁴² and allow for clonal expansion of anergic cells.⁴³ This reinforced our previous sug-

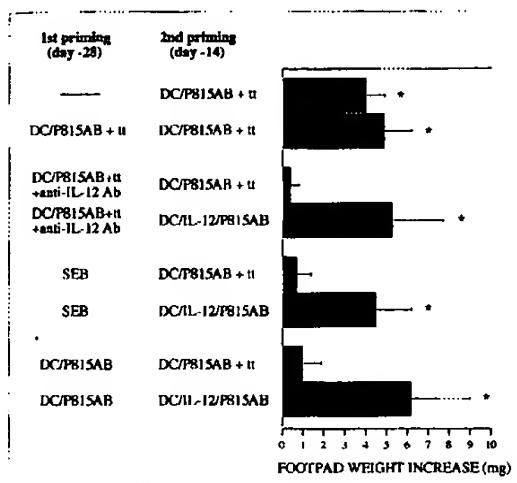


FIGURE 6. Ability of SEB or IL-12 neutralization to block induction of P815AB responsiveness and reversal of this effect by recombinant IL-12. Two weeks before an otherwise effective priming with double-pulsed DC (DC/P815AB + tt), mice were treated either with SEB (50 µg) or with a combination of double-pulsed DC and anti-IL-12 antibodies (a total of 500 µg of affinity purified sheep anti-mouse IL-12 polyclonal antibody). An additional group received, instead, a tolerogenic priming with P815AB (alone)-pulsed DC (DC/P815AB). In studying the possible reversal of each of these tolerogenic primings, the second priming was performed in parallel with DC pre-exposed *in vitro* to IL-12 prior to P815AB pulsing. The DTH reaction was measured two weeks (day 0) after the second priming. * $p < 0.05-0.01$ (peptide challenge vs. saline).

gestion that the production of IL-2 by CD4⁺ cells may be a critical factor in initiating or restoring T cell responsiveness to P815AB. In line with this hypothesis, Figure 7 shows that the restoring of DTH responsiveness to P815AB by recombinant IL-12 was associated with a dramatic increase in IL-2 production by CD4⁺ cells recovered from mice at the time of the skin test assay.

However, an as yet unresolved issue raised by the above findings relates to the mechanisms underlying the differential ability of P815AB and helper peptides to fully activate the APC function of DC and to result in effective recognition of P815AB by CD4⁺ cells. A likely possibility is that the helper epitope of P815AB is suboptimal, because both length and sequence of a peptide contribute to the stability of binding to class II molecules.⁴⁴ Our data on the limited persistence of MHC-P815AB complexes *in vitro* suggest that

poor stability of these complexes contributes to the inadequate activation of APC function in DC. The co-presence of helper peptides might help to compensate for the poor P815AB binding by increasing IL-12 production. However, once tolerance has been established by an ineffective priming with P815AB (alone)-pulsed DC, the Th peptide-induced production of IL-12 may not be sufficient to overcome unresponsiveness, and exogenous IL-12 would be required.

VIII. P815AB, A TUMOR-ASSOCIATED AND SELF ANTIGEN PEPTIDE, AS A MODEL FOR TUMOR-SPECIFIC SHARED ANTIGENS IN HUMANS

Tumor-specific shared antigens in humans, such as members of the MAGE, BAGE, and GAGE families found in melanoma and other cell types, derive from nonmutated self proteins⁴⁵ and have an expression pattern similar to P815AB (i.e., in testis and placenta). Because the human

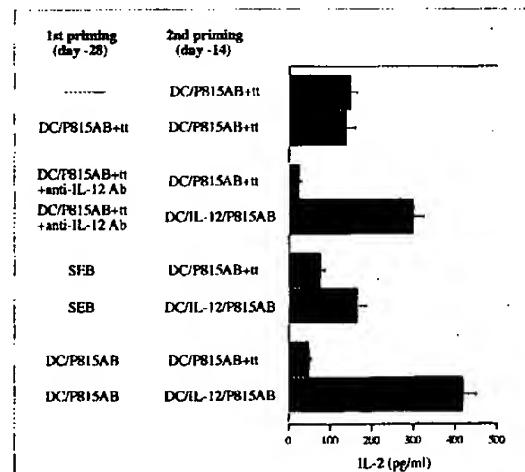


FIGURE 7. Ability of IL-12 to restore IL-2 production by CD4⁺ cells from otherwise P815AB-tolerant mice. A tolerogenic priming with SEB, double-pulsed DC + anti-IL-12, or P815AB (alone)-pulsed DC (see legend to Figure 6) was followed by treatment with IL-12-treated and P815AB-pulsed DC. Two weeks later, at the time of DTH assessment, mice were also used as a source of positively selected CD4⁺ cells to be assayed for IL-2 production *in vitro* in response to 48 h stimulation with P815AB and APC. Results are expressed as pg/ml in supernatants (mean \pm SD).

germline expressing such antigens in testis does not possess classic MHC class I molecules, self-reactive responses are unlikely to develop even in cancer patients specifically immunized against one of these antigens.⁴⁵ In male mice immunized with P815AB cells so as to elicit strong CTL responses, no inflammation of the testis or impairment of fertility is observed.⁴⁶ In our experiments, the combined effects of P815AB and Th peptides in promoting reactivity in naive male mice, but not in deliberately tolerized animals, indicate that the two conditions may be qualitatively different, suggesting that poor immunologic accessibility, rather than tolerance to P815AB, operates in adult life to prevent auto-reactivity to this peptide. The maintenance of anergy is, in fact, an active process requiring the presence of antigen,⁴² which is compatible with our finding that unresponsiveness to P815AB was reverted at about two months after a tolerogenic priming. Nonetheless, at variance with the effect of the Th peptide, recombinant IL-12 is highly effective in reverting unresponsiveness induced by a tolerogenic priming, thus demonstrating that IL-12 can both effectively prevent and revert anergy to P815AB. Although it is clear from our current studies that T cells with potential reactivity to P815AB are present in adult mice and that they can be driven into reversible anergy, we are currently evaluating the specific responsiveness and possible modulation of transgenic mice expressing P815AB in most adult tissues.

CONCLUSIONS

Taken together, the data reviewed in the present article demonstrate that a tumor-associated and self antigen peptide, expressed by murine mastocytoma cells, can result in a reversible state of antigen-specific T cell anergy under priming conditions that are unsuitable for initiating functional responses *in vivo*, such as host transfer with peptide-pulsed DC without added adjuvanticity. The anergic state involves unresponsiveness in CD8⁺ cells as detected by skin test assay *in vivo*, and suppression of IL-2 production by CD4⁺ cells *in vitro*. Endogenous IL-12 has a key role in determining the outcome of an *in vivo* priming with the peptide, acting as both an adju-

vant and an inhibitor of anergy induction. Exogenous IL-12 not only prevents the onset of tolerance, but is also able to revert unresponsiveness to the tumor peptide. The complex effects of IL-12 in our model system may include improved presentation of class II-restricted epitopes of P815AB by accessory cells and a direct action on CD4⁺ cells to increase antigen recognition and to allow for clonal expansion and IL-2 production. This would imply that the effects of IL-12 relate not only to the potentiation of an intrinsically deficient Th1 response, but also to regulation of a balance between activation and tolerogenic signals acting on T cells. These data may offer new insights into the role of dendritic cells, IL-12, and CD4⁺ lymphocytes in the generation of class I-restricted antitumor immunity. They may also be useful in implementing effective vaccination strategies using tumor peptides in humans.

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Special Article

Morphology, function and pathology of follicular dendritic cells

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The precise ultrastructural morphology and functions in reactive conditions of lymphoid follicles (LF) and dendritic cells, including follicular dendritic cells (FDC) are reviewed; as well as the pathognomonic role of FDC in some disease conditions and finally, the cellular origin of FDC. In reactive conditions, FDC in each of the five follicular zones have distinct ultrastructural features, reflecting the different three-dimensional structures and functions of these zones. The FDC framework may be supported by some characteristic factors, including desmosome-like junctions between FDC and the expression of fibronectin and laminin receptors and caldesmon on FDC. FDC, especially in the light zone, express various cytokine receptors, but produce only one cytokine, TGF- β . The outer zone may not only be a cellular pathway in the LF, but may also provide a site for germinal center B cell proliferation, and the FDC-lymphocyte cluster is not the site of germinal center B cell division. In patients with autoimmune diseases, such as Hashimoto's thyroiditis and rheumatoid arthritis, FDC may be in a hyperfunctional state, whereas those in patients with immunosuppressive disorders, such as Kimura's disease and AIDS, may be in a dysfunctional state. FDC may be derived from fibroblastic reticulum cells in lymphatic tissues rather than in bone marrow cells. The data discussed in this review provide fascinating insight into the roles of FDC, which are intimately related to the migration, proliferation, cell selection and differentiation of B cells in secondary LF.

Key words: autoimmune disease, cellular origin, follicular dendritic cell, function, lymphoid follicle, malignant lymphoma, ultrastructure

Both intrinsic and extrinsic antigens provoke immune responses, which involve directly groups of immunocompetent cells, including T and B cells, macrophages and dendritic cells (DC). When all these cells are activated, various cytokines are produced and released that induce activation and proliferation of the same and other types of cell, finally resulting in them playing roles in humoral and cellular immu-

nity. Recently, DC acting as antigen-presenting cells (APC) were recognized as playing an important role in initiating the immune response.^{1,2}

So far, research into lymphatic tissues has been focused mainly on the morphology, function and pathology of lymphocytes and only a few researchers have been interested in follicular dendritic cells (FDC). With recent advances in immunology, however, the significance of this cell as an APC has been pointed out,³ and in the absence of FDC impairment of humoral immunity and failure of production of specific anti-bodies against extrinsically invading antigens have occurred.

In this review, we briefly describe lymphoid follicles (LF) and DC, including FDC, and then outline their precise ultrastructural morphology and functions in reactive conditions, the pathognomonic role of FDC in some disease conditions and finally, refer to the cellular origin of FDC. The data discussed in this review provide a fascinating insight into the roles of FDC, which are intimately related to the migration, proliferation, cell selection and differentiation of B cells in secondary LF.

A BRIEF REVIEW OF LYMPHOID FOLLICLES AND DENDRITIC CELLS

Lymphoid follicles

Numerous lymph nodes are located throughout the body. Afferent lymphatics enter, one efferent lymphatic exits and a meshwork of lymphatic sinuses extends throughout each entire lymph node (Fig. 1). A lymph node is composed largely of three areas: the cortex, the paracortex and the medulla. The cortex possesses primary and secondary LF called 'B-cell-dependent areas', which are occupied by a fine meshwork of FDC and accumulated B cells (Fig. 2).⁴ The paracortex and interfollicular areas are called 'T-cell-dependent areas' and the medulla contains the lymphatic sinuses and predominantly plasma cells. Antigens derived

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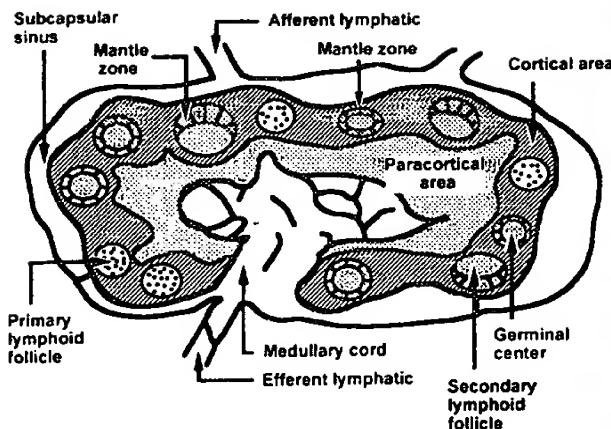


Figure 1 Lymph node structure.

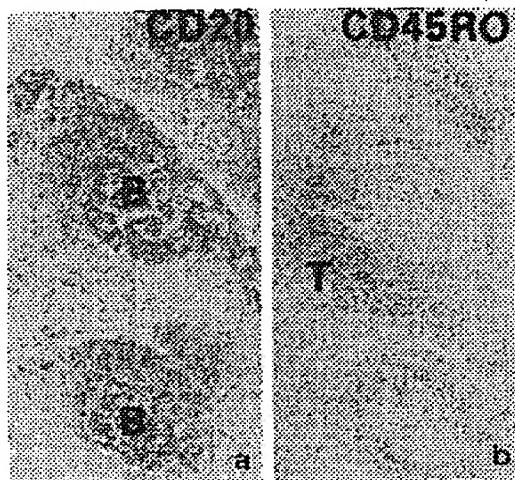


Figure 2 (a) B-cell- (CD20) and (b) T-cell (CD45RO)-dependent areas in serial sections of a lymph node. B cells are located predominantly in the lymphoid follicle and scattered in the interfollicular areas, whereas T cells are located predominantly in the interfollicular areas and scattered in the lymphoid follicle. B, B-cell-dependent area; T, T-cell-dependent area (immunoperoxidase method, counterstained with methylgreen).

from primary inflammatory lesions are transported to draining lymph nodes via the afferent lymphatics and provoke the evolution of antigen-specific antibody secreting cells and memory B cells.⁵ These cells leave the lymph nodes via the afferent lymphatics, pass through large lymphatic canals, such as the thoracic duct, then circulate in the blood and finally take up residence in the primary inflammatory lesions, bone marrow, gut-associated tissues and so on.⁶

Light microscopic examination of tissue sections stained with hematoxylin and eosin (HE) reveals that enlarged secondary LF have a central pale germinal center (GC) and a

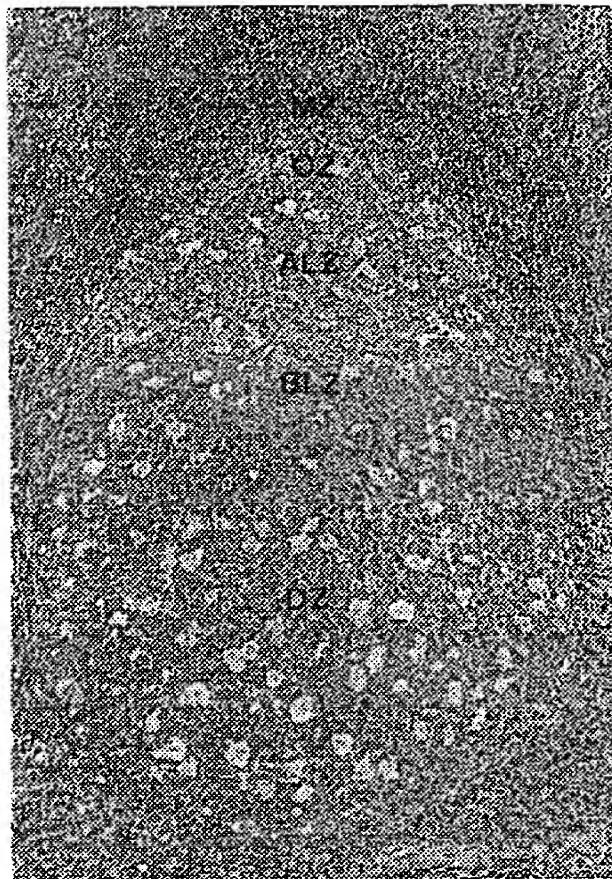


Figure 3 Tonsillar lymphoid follicle. MZ, mantle zone; OZ, outer zone; ALZ, apical light zone; BLZ, basal light zone; DZ, dark zone (HE).

peripheral, crescent-shaped mantle zone (MZ) densely packed with many small resting lymphocytes. Primary LF lack a GC but contain many small lymphocytes corresponding to the MZ of secondary LF. FDC reside in both primary and secondary LF. MacLennan *et al.*⁵ and Hardie *et al.*⁶ divided the GC into four zones: a ring- or doughnut-like outer zone (OZ) in the periphery of the GC, an uppermost pale light zone (LZ), which is subdivided into an upper apical light zone (ALZ) and a lower basal light zone (BLZ), and the lowest dark zone (DZ) at the bottom of the LF (Figs 3,4). LF in all tissues, including not only the so-called 'lymphatic tissues', such as the lymph nodes, tonsils, spleen and Peyer's patches, but also chronically inflamed non-lymphatic tissues, have similar follicular constitutions when longitudinal sections across their central portions are examined. However, recently Brachtel *et al.* pointed out structural differences between tonsillar and lymph node follicles.⁷

The GC is the site of oligoclonal growth and differentiation of memory B cells and plasmablasts with activity against

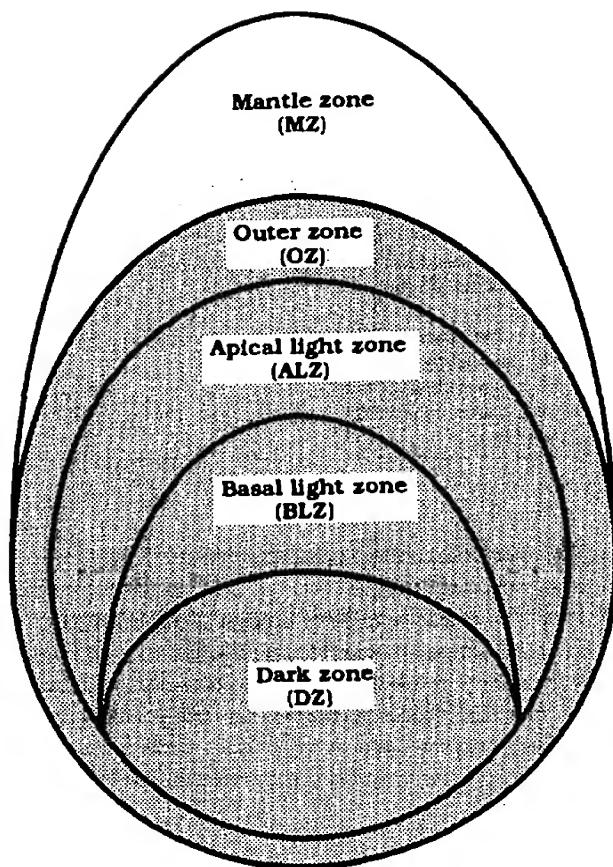


Figure 4 Diagram of a secondary lymphoid follicle. (■) Germinal center.

antigens.⁸⁻¹⁰ Activated B cells migrate into the GC and then undergo somatic mutation in the variable region of their immunoglobulin (IgV-region) gene. B cells make contact with antigens retained on the surface of FDC and B cells with a low affinity for the antigen die immediately by apoptosis, whereas those with a high affinity survive and continue to differentiate into memory B cells expressing membrane (m)Ig. These memory cells are relatively long-lived and circulate in the peripheral blood, enabling the body to respond rapidly to tiny amounts of extrinsically invading antigens of the same type.

Dendritic cells

Briefly, DC are non-lymphoid APC distributed widely throughout the body and are clearly distinguishable from macrophages by their lack of both phagocytic activity and their capacity to act as effector cells. Morphologically, they have complex dendritic cytoplasmic projections, one or more lob-

ulated nuclei and a clear cytoplasm with sparse organelles. DC are indispensable for the induction of an immune response,¹⁰ and they are classified into two groups: B- and T-cell-associated DC (Table 1).¹¹ The former are identical to FDC, which are APC for B cells, and the latter are APC for T cells and are divided further into three groups: (i) non-lymphatic tissue DC, including epidermal Langerhans cells, DC in connective tissues and those of unknown origin (indeterminate cells in the dermis, dermal Langerhans cells and Granstein cells in mice); (ii) DC in circulatory blood and lymphatic fluids, generally called veiled cells (dendritic leukocytes); and (iii) lymphatic tissue DC, called interdigitating cells, in T-cell-dependent areas.

Historical review of follicular dendritic cells

In the 1950s LF were reported¹² to trap antigens and immune complexes (IC) (Table 2). Kojima *et al.* pointed out the significance of desmosome-like junctions of non-lymphoid (desmodendric) cells in the GC¹³ and Chen *et al.* were the first to propose the name 'FDC',¹⁴ which is now the accepted term for this type of cell. In advance of the general acknowledgement of the unique functional significance of FDC in immune responses, Tew *et al.* were the first to define this type of cell;¹⁵ that is, FDC are identified in tissue sections of LF, they trap and retain antigen-antibody complexes on their surfaces and express Fc and C3 receptors, but they lack Ia and Thy-1 antigens and surface immunoglobulin.

In 1983, Imai *et al.* divided FDC into two types, according to whether or not they had a labyrinth-like structure.²¹ One type is characterized by specialized, abundant, labyrinth-like structures and numerous well-developed desmosome-like junctions and is located especially in the LZ, whereas the other lacks labyrinth-like structures and has few, poorly developed desmosome-like junctions. Later in the 1980s,

Table 1 Classification of dendritic cells

B-cell associated dendritic cells
Follicular dendritic cells
T-cell associated dendritic cells
Non-lymphatic tissue dendritic cells
Langerhans cells
Connective tissue dendritic cells
Unknown-origin dendritic cells
Indeterminate cells
Dermal Langerhans cells
Granstein cells in mice
Dendritic cells in circulatory fluids
Veiled cells (dendritic leukocytes)
Lymphatic tissue dendritic cells
Interdigitating cells

Table 2 Historical review of follicular dendritic cell (FDC)

1950 Kaplan <i>et al.</i> ¹²	Antigen trapping in lymphoid follicle
1962 Mellows & Brzarsko ¹³	Immune complex trapping in germinal center
1968 Nossal <i>et al.</i> ¹⁴	Follicular reticular cell, antigen-retaining reticular cell
1968 Hanna & Szakal ¹⁵	Antigen-retaining reticulum cell
1978 Kojima <i>et al.</i> ¹⁶	Desmo-dendric cell
1978 Lennert ¹⁷	Dendritic reticulum cell
1978 Chen <i>et al.</i> ¹⁸	FDC
1982 Tew <i>et al.</i> ¹⁹	Definition of FDC
1982 Humphrey & Grennan ²⁰	Isolation of FDC from mouse spleen
1983 Imai <i>et al.</i> ²¹	FDC with/without labyrinth structure
1983 Naiem <i>et al.</i> ²²	Production of a monoclonal antibody (R4/23)
1984 Lilet-Leclercq <i>et al.</i> ²³	Isolation of human FDC
1992 Clark <i>et al.</i> ²⁴	Long cultivation of FDC with GM-CSF
1993 Imai <i>et al.</i> ²⁵	Non-bone marrow origin
1994 Lindhout <i>et al.</i> ²⁶	EB virus-transformed human FDC-like cells

some monoclonal antibodies that reacted relatively specifically with FDC were prepared and were found to be very useful for identifying FDC in non-neoplastic and neoplastic lymphatic tissues.^{22,27-29} The next success was the establishment of methods for isolating FDC from FDC-rich lymphatic tissues such as tonsils.^{30,31} Recently, methods for the long-term cultivation of FDC obtained from FDC-lymphocyte clusters and of isolated FDC in the presence of additional cytokines, such as granulocyte-macrophage colony stimulating factor (GM-CSF), were developed.²⁴ Although several studies of the cellular origin(s) of FDC have been reported the results are rather controversial.^{25,32-35}

Proposed new definition of follicular dendritic cells

As discussed earlier, the first definition of FDC was suggested in 1982,¹⁹ but, over the past 10 years, a great deal of successful work has contradicted some of this definition. Therefore, a new definition of this type of cell is proposed here (Table 3).

Our new definition is classified into two major categories: the broad and the narrow sense. The former describes the FDC anywhere in the LF, including those in the DZ, and in the lower half of the OZ and MZ, and they can be identified using immunohistochemical and electron-microscopic techniques. The latter describes the FDC that are in the LZ, and in the upper half of the OZ and MZ directly beyond the ALZ (the so-called 'cap area'), and these FDC can be identified

Table 3 New definition of follicular dendritic cells (FDC)

Broad sense
Light-microscopic: Cells forming reticular meshwork in the lymphoid follicles positive for anti-FDC antibodies
Electron-microscopic: Cells having desmosome-like junctions between same types of cells in the lymphoid follicles
Narrow sense
Electron-microscopic: Cells having labyrinth-like structures and desmosome-like junctions between same types of cells in the lymphoid follicles
Functional:
Cells in the lymphoid follicles to trap and retain immune complex for a long time
Cells to form clusters with B cells
Cells positive for anti-FDC antibodies to present antigen to B cells

by their ultrastructural findings and by the results of functional assays, including antigen-trapping and long-term retention abilities, the capacity to form cellular clusters with B cells *in vivo*, and antigen-presentation to B cells *in vitro*. However, it is necessary to identify cells that simultaneously express markers of FDC. Needless to say, this definition is applicable to cases involving disease conditions, such as malignant lymphomas, in non-lymphatic and lymphatic tissues.

MORPHOLOGY OF FOLLICULAR DENDRITIC CELLS IN REACTIVE LYMPHOID HYPERPLASIA

Ultrastructural findings of follicular dendritic cells

Enlarged secondary LF in tonsils obtained from children with chronic tonsillitis have the five easily recognizable follicular zones, in the following order from the tonsillar crypts to the deeper sites: MZ, OZ, ALZ, BLZ and DZ.

Here, we present the distinct ultrastructural features of FDC found in the MZ, OZ, DZ, BLZ and ALZ, in that order, obtained using conventional transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Furthermore, two modified SEM techniques were employed in order to remove the surrounding lymphocytes and thus enable the entire cell bodies of the FDC to be discerned easily. One is an etching method, in which Epon-embedded semithin sections (6–8 µm thick) were treated with a saturated solution of KOH in absolute ethanol for 3 days at room temperature. The other is the trypsin treatment method, in which trimmed fresh tonsillar tissues were fixed first in 4% w/v paraformaldehyde for 6 h at 4°C, rinsed with 0.01 mol/L phosphate-buffered saline and then 200-µm-thick slices were cut with a vibratome. The sliced tissues were treated with 0.5% w/v trypsin and 0.001% w/v CaCl₂ in 0.01 mol/L phos-

phate-buffered saline for 1 h at 37°C. After rinsing with 0.01 mol/L phosphate-buffered saline, tissues were refixed with 1.25% v/v glutaraldehyde and 1% w/v osmic acid, routinely embedded in Epon, and ultrathin sections were observed using SEM.

Mantle zone

In longitudinal tissue sections, the MZ appears as a crescent shape along the greater axis of the LF. Unlike the other zones, it is composed of a relatively monotonous accumulation of small lymphocytes with some intermingled FDC (Fig. 5). The MZ contains recirculating mIgM⁺ and mIgD⁺ small resting B cells, which are precursors of the centroblasts in the DZ.^{5,6,38} These cells are also CD10⁻, CD20^{dim}, CD23⁺, CD38⁻, CD39⁺, CD44⁺, CD71⁻ and negative for peanut agglutinin. MZ lymphocytes are polyclonal with germ line IgV-region genes and possess bcl-2 protein and its mRNA.³⁷⁻⁴⁰ The FDC network at the top of the LZ dips into the MZ forming a crescent-like cap area, which is the front area for trapping IC.

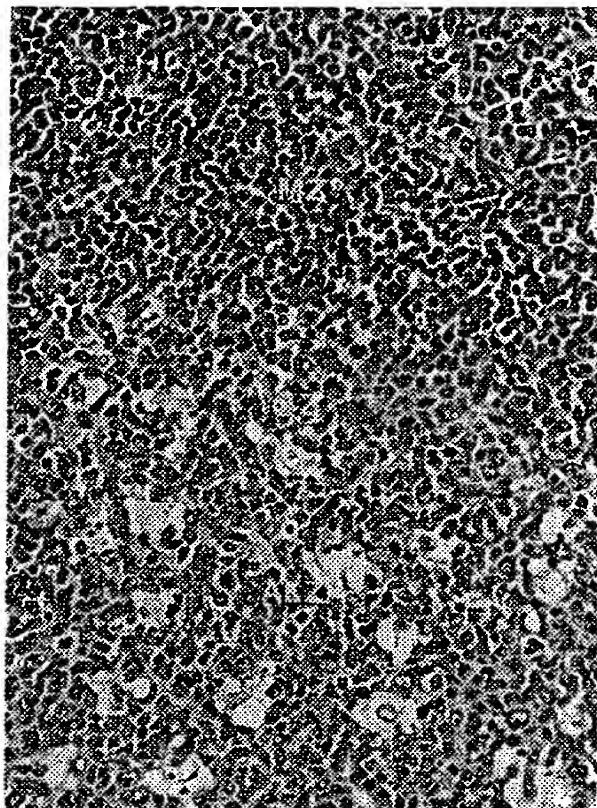


Figure 5 Light microscopy features of the mantle (MZ), outer (OZ) and apical light (ALZ) zones. The MZ is densely packed with small lymphocytes, the OZ contains a variety of cell types and the ALZ consists predominantly of medium-sized centrocytes. TBM are present in the ALZ (HE).

FDC are easily recognized on toluidine-blue-stained Epon semithin sections, showing one or more large pale nuclei, and those in the lateral MZ have cell bodies with arcs protruding towards the top of the MZ (Fig. 6).

Conventional TEM shows that the MZ in the cap area is sometimes found to contain FDC-lymphocyte clusters composed of one or more FDC connected to each other by desmosome-like junctions and some lymphocytes tightly encircled by the relatively smooth, slender cytoplasmic extensions of the FDC (Fig. 7). Unlike those in the LZ, the

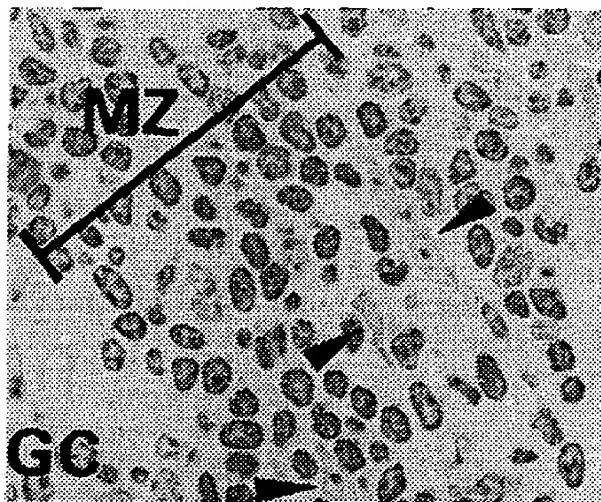


Figure 6 The lateral MZ is composed of dense accumulations of lymphocytes and FDC (arrowheads) running parallel to each other. GC, germinal center (Epon-embedded semithin section, toluidine blue stain).

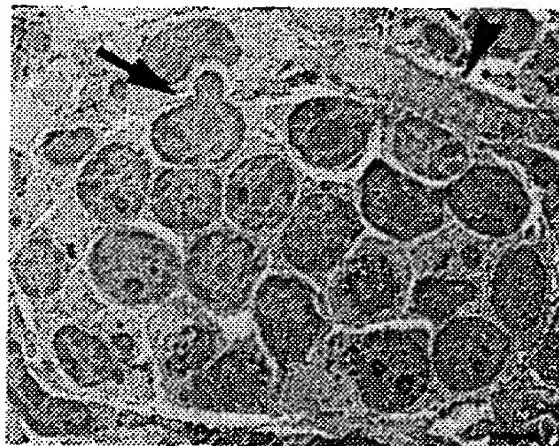


Figure 7 Transmission electron microscopic features of a FDC-lymphocyte cluster in the MZ. One FDC (arrowhead) loosely encircles 22 lymphocytes, and a lymphocyte is leaving a cluster (arrow). Bar = 2 μm.

FDC in the MZ lack these complicated, villous, cytoplasmic processes, which extend into the intercellular spaces of the encircled lymphocytes in the clusters. Occasionally, lymphocytes leaving the clusters are also found. FDC that do not form clusters have only a few simple cytoplasmic extensions projecting towards the intercellular spaces between adjacent lymphocytes (Fig. 8). FDC in the lateral MZ (outside the cap area) have desmosome-like junctions, but those in the clusters rarely do, and no FDC anywhere in the MZ have typical labyrinth-like structures. Lymphocytes in the lateral MZ are packed in a multi-lamellar arched pattern, partitioned off by FDC arranged parallel to each other (Fig. 9), suggesting that the FDC should be arranged to control the lymphocyte traffic.

A low-power SEM view of a trypsin-treated section demonstrates an extensive reticular meshwork stretching throughout the entire secondary LF. The meshwork in the LZ is looser than those in the MZ and DZ, but the LZ contains more large hollows, which may be the prototype of the FDC-lymphocyte clusters, than the MZ and DZ. A high-power SEM view shows that the whole MZ has a complex reticular meshwork and the MZ in the cap area contains some small hollows indicative of FDC-lymphocyte clusters (Fig. 10). At the bilateral lower apices of the crescent-shaped MZ, spindle-shaped FDC with desmosome-like junctions but no labyrinth-like structures run parallel to each other with lymphocytes between them (Fig. 11). Both conventional TEM and trypsinized SEM methods reveal that these apices may have two cellular pathways defined by the FDC arrangement: one is a path connecting the MZ and/or OZ to the extrafollicular area and the other is a path into the OZ itself.

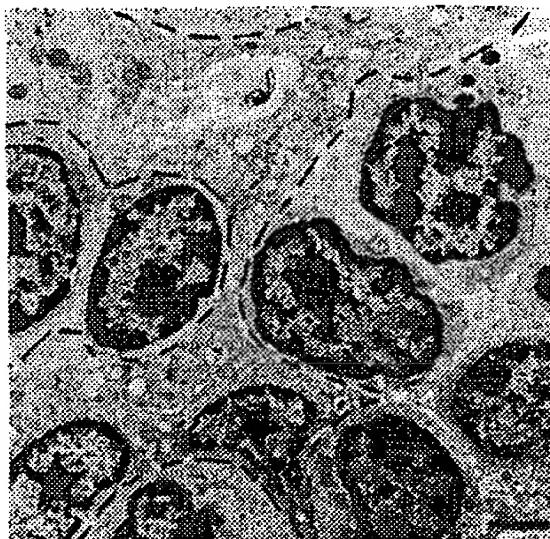


Figure 8 Transmission electron photomicrograph of a FDC in the MZ. This binucleate FDC (*) has an umbrella-like outline (---). Bar = 1 μ m.

Outer zone

The OZ is a narrow ring-like structure surrounding the outer rim of the GC. The upper half of this zone lies between the MZ and ALZ and the lower half between the DZ and extrafollicular area, including the T-cell-dependent area (Fig. 5). Characteristically, lymphocytes in this zone express CDw75 more strongly than the other GC cells.⁶ The OZ contains rather heterogeneous cell types, including lymphocytes similar to centrocyte-like cells, Ki-67⁺ blasts and plasma-cell-like cells. The centrocyte-like cells may migrate back into the DZ to be changed and re-selected. CD4⁺ Th2 cells also accumulate at the junction between the MZ and GC,^{4,42} and the CD4:CD8 ratio at this site is 12:1, compared with 2:1 in the T-cell-dependent areas.⁴³ The FDC network in the OZ is somewhat loose and FDC express little or no Fc ϵ RII (CD23).^{5,44} Electron microscopically, FDC with long slender cytoplasmic extensions run parallel to each other forming an arch, like those in the MZ outside the cap area, following the ring-shape of the OZ itself. These FDC have desmosome-like junctions, but do not have the typical labyrinth-like structure nor do they form FDC-lymphocyte clusters.

Dark zone

Large centroblasts in the DZ have little or no mlg or cytoplasmic Ig, and strongly express CD77 antigen, a marker of activated B cells.⁶ The cell cycle of centroblasts lasts about 7 h and they generate numerous centrocytes for less than 24 h.⁵ Frequent mitotic figures are observed, indicating that

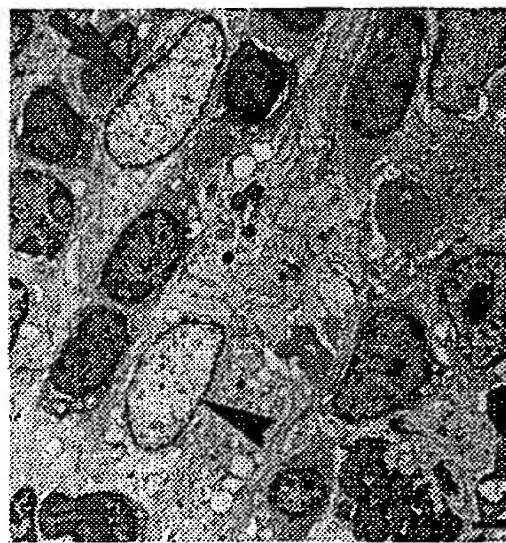


Figure 9 Transmission electron photomicrograph of the lateral MZ. Note the parallel arrangement of the FDC (arrow, arrowhead) providing a cellular pathway for the lymphocytes. Bar = 1 μ m.

the DZ is the centroblast proliferation site (Fig. 12). Some tingible body macrophages (TBM), a unique and true macrophage within the GC, are found phagocytosing apoptotic bodies derived from GC lymphocytes. The FDC network is widely, but loosely, distributed in this zone.

TEM reveals that the FDC in this zone have desmosome-like junctions and less well-developed cytoplasmic extensions than those in the LZ, but lack the atypical labyrinth-like structure (Fig. 13). No distinct arrangement of FDC for the purpose of controlling lymphocytic traffic is ever observed in

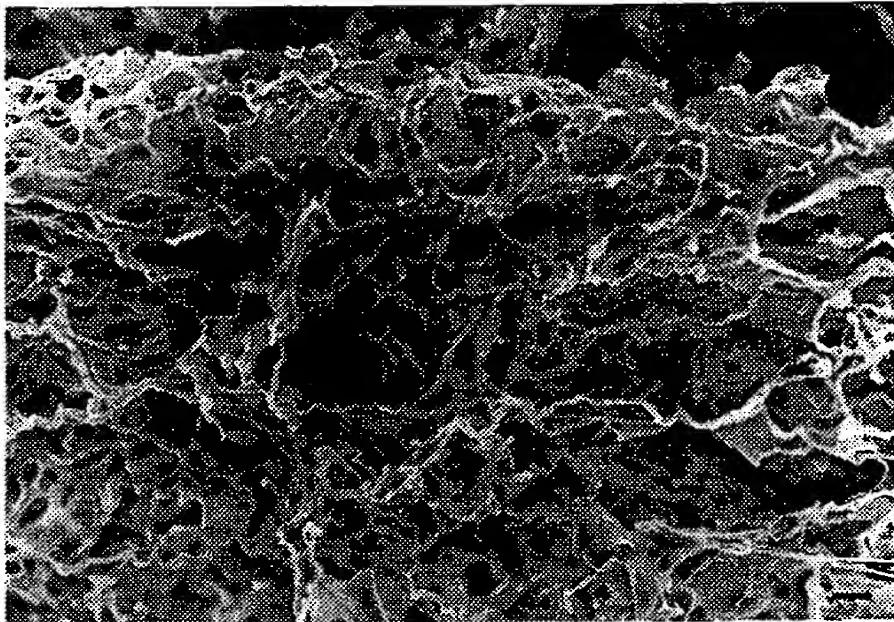


Figure 10 Modified scanning electron microscopic features of the top of the MZ. Note the reticular meshwork of FDC. Scattered openings suggestive of FDC-lymphocyte clusters are present. Bar = 0.1 μ m.

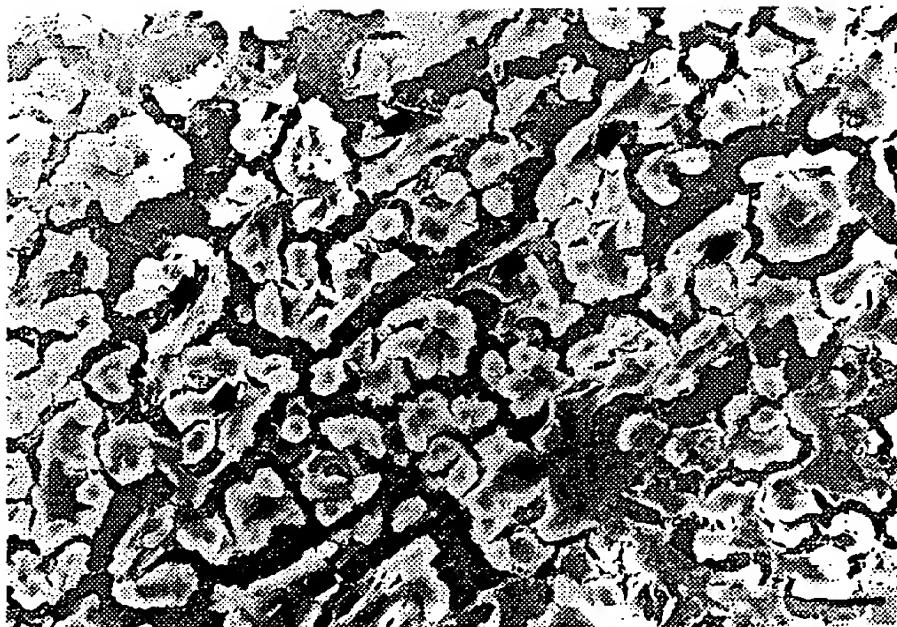


Figure 11 Scanning electron microscopic view of the lateral MZ. Note the parallel arrangement of FDC (arrows) providing a cellular pathway for the lymphocytes. Bar = 0.1 μ m.

the OZ under the SEM. The FDC cell bodies are relatively plump and although the FDC encircle centroblasts rather loosely, they do not form FDC-lymphocyte clusters.

Basal light zone

The BLZ contains large centrocytes of the same size as centroblasts and frequent TBM, but mitotic figures are infrequent (Fig. 12).⁴⁵ The FDC are pyroninophilic, possess one or more well-developed nuclei, and express complement receptor (CR) type 2 (CD21) and intercellular adhesion molecule (ICAM)-1 (CD54), but little or no CD23.^{5,44,48}

Most centrocytes leave the GC over a period of 1 or 2 days to become memory B cells or plasmablasts, or die as a result of apoptosis.^{5,47} Apoptotic cells are found more frequently in the BLZ than in the other zones in the GC. The most important factors for cell selection are the degree of down-regulation of mIg receptors on centrocytes after somatic mutation and the probability of making contact with antigens retained on the FDC surfaces.⁴⁸

TEM reveals well-organized FDC characterized by distinct labyrinth-like structures (Fig. 14) and desmosome-like junctions (Fig. 15), and such FDC are found frequently in this zone. Occasional cytoplasmic extensions of the FDC are twisted

around fibers adjacent to blood vessels. A high-power TEM view reveals that the junctions are composed of two thick lines, called trilaminar cell membranes, sandwiching flocculent materials and bilateral fine filaments (Fig. 16). Less frequently, junctions with intercellular gaps instead of flocculent materials are found. Unlike those in the MZ, OZ and DZ, FDC in the entire LZ have prominent slender cytoplasmic extensions that bind to each other with many desmosome-like junctions, resulting in the establishment of fine reticular meshworks that surround packed centrocytes. Interestingly, a high-power TEM view revealed that each centrocyte is surrounded by many minute villous cytoplasmic extensions of FDC, suggesting active cell-to-cell interaction between centrocytes and FDC. This interaction provides a useful tool for presenting antigens to centrocytes and establishing communication via cytokines and their receptors. Modified SEM techniques reveal that extremely complicated, villous cytoplasmic extensions of FDC occur frequently. The BLZ adjacent to the junctions of the MZ, OZ and DZ, contains a relatively coarser and more extensive reticular meshwork than the other zones, indicating that the BLZ contains large FDC-lymphocyte clusters.

Apical light zone

The apical portion of the LZ is packed with medium-sized centrocytes and infrequently contains mitotic cells and apoptotic bodies (Fig. 5). FDC in the ALZ strongly express CD21.

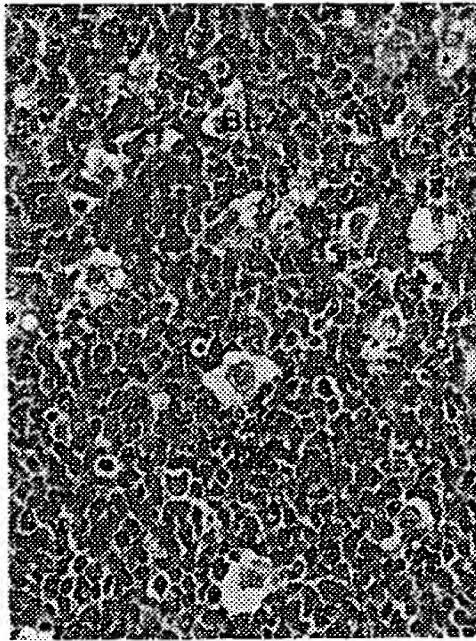


Figure 12 Light microscopy features of the basal light (BLZ) and dark (DZ) zones. The DZ contains dense accumulations of centroblasts with occasional mitotic figures. The BLZ contains relatively loosely packed centrocytes, mitotic figures are rare and scattered TBM are present (HE).



Figure 13 Transmission electron microscopic features of a FDC (★) in the DZ. This FDC has a desmosome-like junction (arrow) but no distinct labyrinth-like structure. Bar = 1 μ m.

CD23 and CD54 and form a denser meshwork through their cytoplasmic projections than those in the other zones.⁴⁹ The GC in patients with rare disease conditions, such as Castleman's disease, contain plasma cells.^{50,51} The ALZ is the site where centrocytes differentiate into memory B cells or plasmablasts, and FDC play a central role in this B cell differentiation. IC retained in the cap area undergo rapid repeated dissociation and re-binding, with a dissociation half-life of approximately 1 h,⁵² and the free antigens induce memory B cell differentiation.⁵³ Conventional TEM demonstrates clearly that this zone, like the BLZ, contains numerous FDC with plentiful labyrinth-like structures composed of fine villous

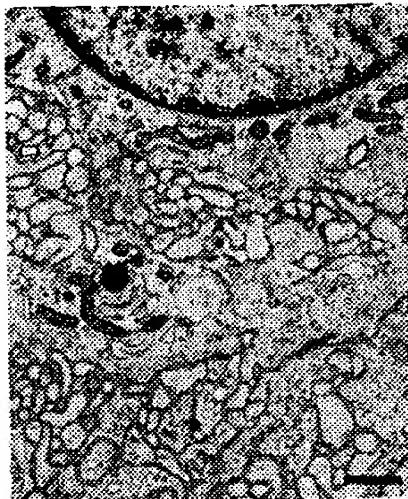


Figure 14 Typical labyrinth-like structure of a FDC in the BLZ. Bar = 2 μ m.

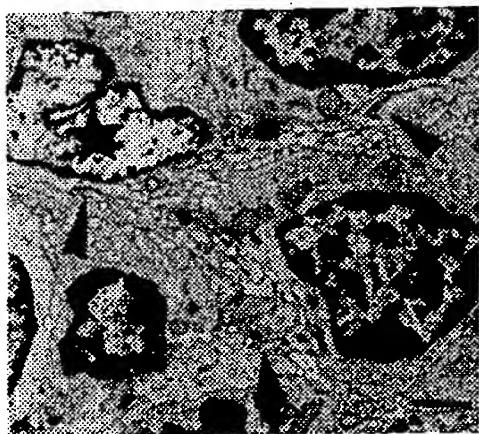


Figure 15 Desmosome-like junction (arrowheads) connecting FDC (*) in the BLZ. Bar = 1 μ m.

projections. Although, in general, electron microscopy shows that the LZ contains numerous well-organized FDC, a variety of types of FDC are found in it (Fig. 17). For example, at the upper margin of the LZ, spindle-shaped FDC, similar to those in the OZ and MZ, are found, and modified SEM techniques frequently reveal typical FDC, like those in the BLZ. One of the most frequently demonstrated types of FDC in the ALZ is shown in Fig. 18.



Figure 16 High-power transmission electron microscopy view of a desmosome-like junction (arrowheads) showing the triaminar cell membrane. Bar = 10 μ m.

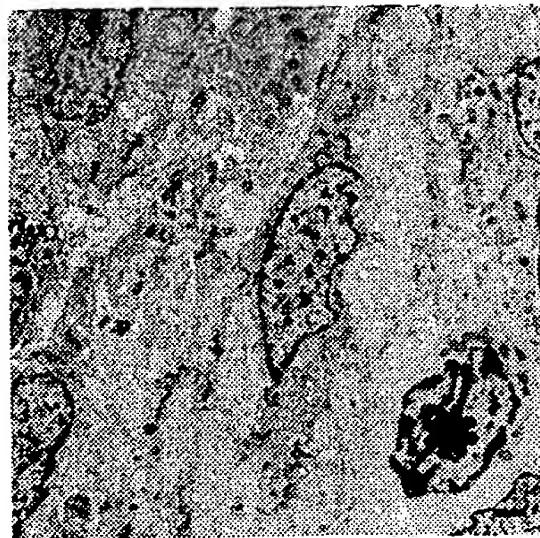


Figure 17 Some FDC in the periphery of the ALZ. These FDC have neither well-developed cytoplasm nor cell processes. (*) Centrocyte. Bar = 1 μ m.

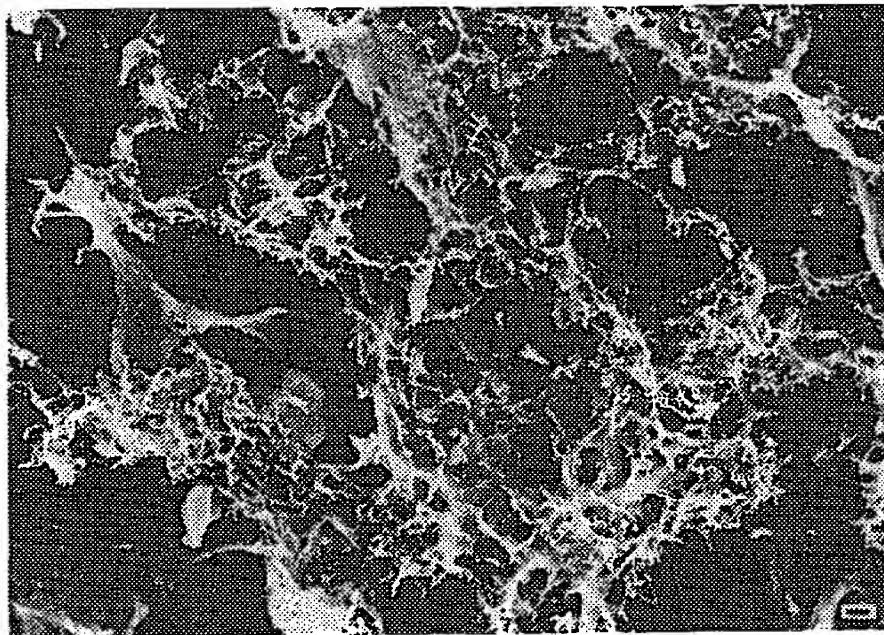


Figure 18 Modified scanning electron microscopic view of typical reticular cytoplasmic extensions of FDC in the ALZ. Bar = 0.4 μ m.

Desmosome-like junctions

We evaluated which types of cells connect to FDC via desmosome-like junctions. Each type of junction on the FDC surfaces shown on TEM photographs was marked with a different color (Fig. 19), which revealed there were no junctions between FDC and lymphocytes, or between the cytoplasmic processes of an individual FDC or an individual lymphocyte or between lymphocytes themselves, indicating that desmosome-like junctions are limited to connecting neighboring FDC.

Fine cytoplasmic extensions in labyrinth-like structures

We identified two basic types of villous cytoplasmic extension using TEM (Fig. 19): one was similar to viral budding with no discernible stalks and the other was the dendritic type with one stalk and subsequent branching cytoplasmic extensions.

Furthermore, we evaluated the complexity of the entire FDC surface, including cytoplasmic extensions. On TEM photographs, the margins of the surfaces of FDC and lymphocytes in the LZ were traced, the perimeter (P) and area (A) of each cell body were calculated and the form coefficient was estimated (Table 4), indicating that FDC surfaces are about 70 times more complex than those of centrocytes, chiefly because the FDC have extremely complicated labyrinth-like structures and intricate villous cytoplasmic extensions.

Conclusion

As described, the ultrastructure of FDC in the five tonsillar follicular zones is heterogeneous, which is attributable to their different three-dimensional structures. FDC in the LZ have more well-developed labyrinth-like structures than those in the other zones, whereas desmosome-like junctions are found on FDC in all five zones. These junctions only occur between neighboring FDC and connection of neighboring FDC by these junctions may build up a complicated framework in the secondary LF. Furthermore, numerous villous cytoplasmic extensions on FDC surfaces provided an environment in which FDC and the surrounding lymphocytes can interact efficiently.

FUNCTIONS OF FOLLICULAR DENDRITIC CELLS

Immune complex trapping and retention

Experiments in mice

Several reports describe IC trapping in the GC,^{54,55} and several authors support the concept that lymphocytes, probably B cells, transfer IC to FDC.⁵⁶⁻⁵⁸ B cells may bind to IC via Fc receptors, move towards the GC and transfer the IC to contacting FDC. However, the results of studies of this process are controversial.⁵⁹

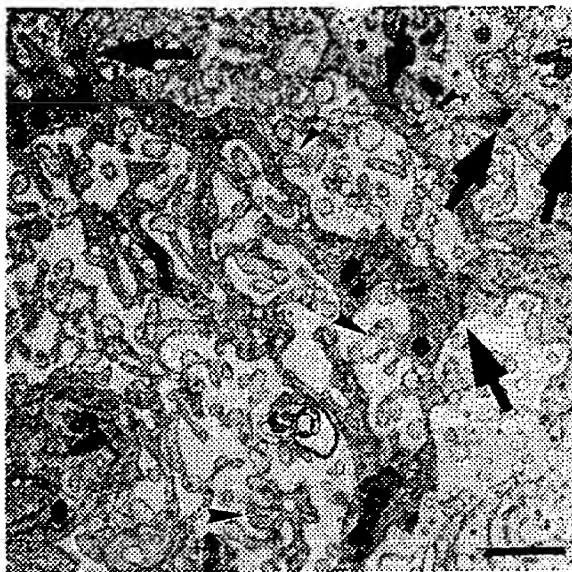


Figure 19 Transmission electron microscopy view of desmosome-like junctions and villous cytoplasmic extensions of FDC. Arrows indicate desmosome-like junctions. Budding-type (short arrowheads) and dendritic-type (long arrowheads) villous cytoplasmic extensions are shown. Bar = 2 μ m.

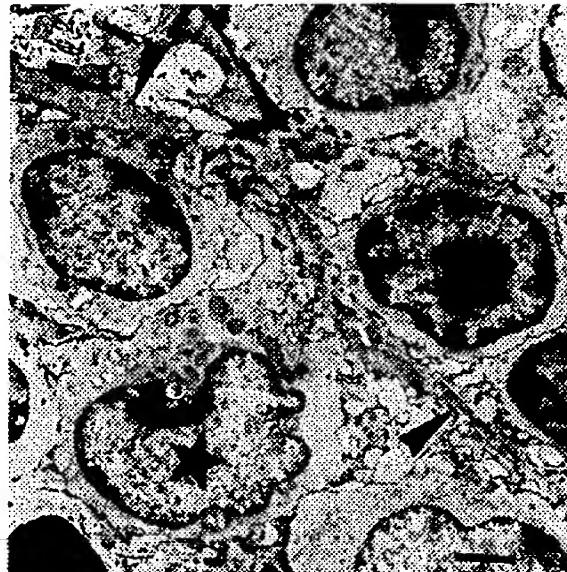


Figure 20 Immune complex trapping by a FDC (★). The immune complex is located predominantly on the labyrinth-like structure of the FDC. Arrowheads indicate extracellular fibers. Bar = 1 μ m.

Table 4 Form coefficient* of follicular dendritic cells (FDC) and B cells

Type of cell	<i>n</i>	Average \pm SEM
FDC	41	109.10 \pm 14.92
B cells	65	1.63 \pm 0.06

*FC = $P^2/4\pi A = 1$; P, perimeter; A, area.

In studies by Imai *et al.*⁵⁴ and Yamakawa *et al.*⁵⁵ the footpads or intestinal lumina of mice were injected with an antigen and IC in the popliteal lymph nodes and Peyer's patches were investigated. The mice were immunized initially with an antigen, such as horseradish peroxidase, and 2 weeks later were immunized again with same antigen. During the early phase (about 30 min to 1 h) after secondary immunization, the antigen was found to be localized in the intercellular spaces on the surfaces of lymphocytes and FDC and along the intervening fibers in the LZ. During the later phase (>3 h) after secondary immunization, the antigen was confined to the cap area composed of the ALZ and the upper parts of the MZ and OZ. Electron microscopy revealed the antigen was localized exclusively on the FDC surfaces, particularly on their labyrinth-like structures (Fig. 20), and occasional centrocytes pinocytosing the antigen were also found (Fig. 21). Once trapped in an LF, the antigen may be retained in it for more than a year. These studies showed that labeled

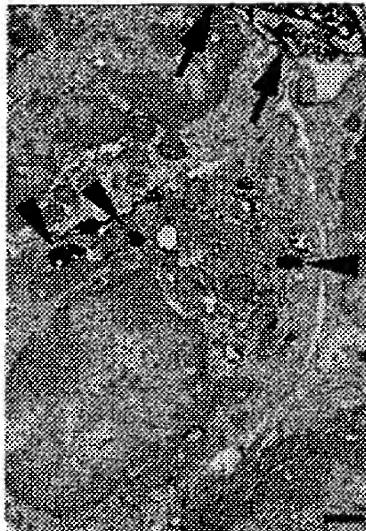


Figure 21 Pinocytosis (arrowheads) of immune complex by centrocytes. Arrows indicate immune complex coating the labyrinth-like structures of FDC. Bar = 2 μ m.

IC are not only trapped on FDC surfaces but they also adhere to fibroblastic reticulum cells (FRC) and collagen fibers in the primary LF. After passing through the surface of the FRC and the fibers in the juxta-LF, IC finally reach the surface of FDC in the GC.

Complement activation in human lymphoid follicles

Both Fc_γRII (CD32) and CR play important roles in trapping and retaining IC.⁶⁰ If complement is not activated, the IC is neither trapped nor retained in the GC and the production of memory B cells responsive to the corresponding T-cell dependent-antigen does not occur. Interactions between the complement system and CR (CD21 and CD35) expressed on B cells and FDC are important for subsequent events in the LF.⁶¹⁻⁶⁴ CD35 on FDC participates in trapping C3b-binding IC, and CD35 and CD21 are essential for production of antibodies against both T-cell dependent and independent antigens involved in the primary response. CD21 binds tightly to iC3b and C3dg and this binding is indispensable for the long-term retention of IC, which is one of the most important roles of FDC.⁶⁵ The precise localization of complement components in the LF, however, is not fully elucidated.

In recent studies^{66,67} we demonstrated the precise localization of the majority of complement components in the classical, alternative and late activation pathways in the LF of human Peyer's patches and appendices (Table 5). Furthermore, their regulatory factors were identified and found to be localized simultaneously. These complement and regulatory factors were limited to the LZ (Fig. 22) and immunoelectron microscopy revealed they occurred mainly on the FDC surface. Although complement activation indeed occurs in the LF, co-localization of regulatory factors prevents lysis of FDC and centrocytes due to complement activation, which does not occur in the MZ or DZ. The follicular localization of complement coincides with that of IC.

In conclusion, long-term IC retention occurs only on the FDC. IC trapping and retention in the LZ depends on complement activation and co-localization of complement regulatory factors is important to protect the cells against lysis. There are several factors common to the complement activation, blood coagulation and fibrinolysis systems and the follicular localization of some factors in the latter two systems have also been demonstrated.^{68,69} Their localization also coincided with those of IC and complement but the significance of this finding is not yet clear.

Adhesion molecules on follicular dendritic cells

Some studies have demonstrated the significance of adhesion molecules in the FDC-to-GC B cell interactions.⁷⁰⁻⁷³ In particular, the interaction via CD54-leukocyte function-associated molecule 1 (CD11a/CD18) and vascular adhesion molecule 1 (CD106)-very late activation antigen 4 (VLA-4, CD49d/CD29) may play a central role. On the other hand, electron microscopy often reveals attachments between the cytoplasmic processes of FDC and extracellular fibers. Gioglini *et al.* also suggested there was a close relationship

Table 5 Localization of complement components and their regulatory proteins in secondary lymphoid follicles

Complements and their regulatory proteins	Light zone (%)	Dark zone (%)	Mantle zone (%)
C1q	100	0	0
C3d	100	0	33.3
Membrane attack complex	100	0	0
C4b binding protein	100	0	0
Properdin	100	0	41.7
CD21	100	100	100
CD35	100	100	100
CD46	100	0	0
CD55	100	0	0
CD59	100	0	0

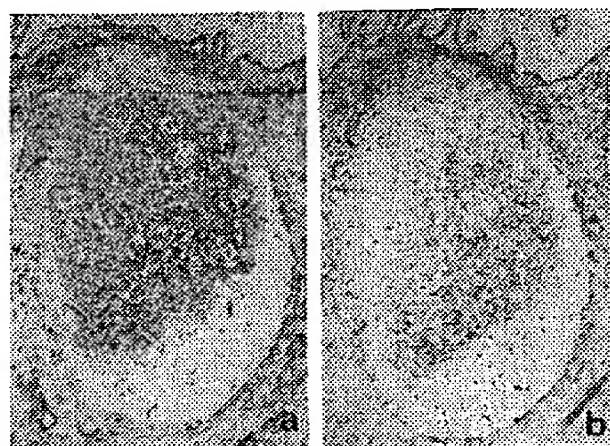


Figure 22 (a) Complement (C3d) and (b) vitronectin in the LZ of a tonsillar lymphoid follicle (immunoperoxidase method, counterstained with methylgreen).

between the cytoplasmic processes of FDC and laminin fibers,^{74,75} but very few reports demonstrating adhesion between FDC and extracellular matrices have been published.

In studies by Ogata *et al.*^{76,77} a magnetic cell sorter⁷⁸ was used with the help of monoclonal anti-FDC antibodies and microbead-labeled anti-mouse Ig antibodies to isolate tonsillar FDC. Tonsillar tissue sections and cytospin preparations containing isolated FDC were immunostained, and the results are summarized in Table 6. FDC in the LF expressed some adhesion molecules, including sialyl-Lewis^x (CD15s), integrin β_1 (CD29), VLA- α 3 (CD49c), VLA- α 5 (CD49e), VLA- α 6 (CD49f), CD54, CD80 and CD106, but ICAM-3 (CD50) was only expressed on FDC in the LZ (Fig. 23). Expression of CD50, CD54 and CD106 on FDC was found to be essential for binding to B cells, presumably for FDC-lymphocyte cluster formation. Immunostaining experiments also indicated that FDC may express both fibronectin and laminin receptors to bind to extracellular matrices and to both reticulin and laminin fibers, respectively. In an attempt to clarify this, a frozen-section binding assay was carried out using isolated, fluores-

cence-labeled FDC.⁷⁷ Pre-treatment of FDC with anti-CD29, CD49e or CD49f significantly inhibited their binding to the LF on the tonsillar sections, indicating that FDC possess fibronectin and laminin receptors (Table 7).

In conclusion, FDC express adhesion molecules essential for binding to B cells and possess fibronectin and laminin receptors, but no vitronectin, fibrinogen or collagen receptors. Binding between the cytoplasmic extensions of FDC and fibers may play an important role in building up and maintaining the three-dimensional framework in the LF.

Ca²⁺-binding proteins in lymphoid follicles

It has been reported that only a few Ca²⁺-binding proteins, such as S-100 protein, annexin VI and calbindin D, are

Table 6 Localization of adhesion molecules on follicular dendritic cells⁷⁷

Adhesion molecules	CD code	MZ	OZ	DZ	BLZ	ALZ
Mac-1	CD11b	+	+	+	++	++
Sialyl-Le ^a	CD15s	+	+	+	+	++
CD22	CD22	+	+	+	++	++
Integrin- $\beta 1$	CD29	+	+	+	++	++
CD40	CD40	++	++	++	++	++
VLA- $\alpha 3$	CD49c	-	-	-	+	+
VLA- $\alpha 5$	CD49e	-	-	-	+	+
VLA- $\alpha 6$	CD49f	-	-	-	+	+
ICAM-3	CD50	-	-	-	+	+
ICAM-1	CD54	+	+	\pm	++	++
B7	CD80	++	++	++	++	++
VCAM	CD106	+	+	+	++	++

MZ, mantle zone; OZ, outer zone; DZ, dark zone; BLZ, basal light zone; ALZ, apical light zone.

-, Negative; \pm , often positive; +, weakly positive; ++, strongly positive.

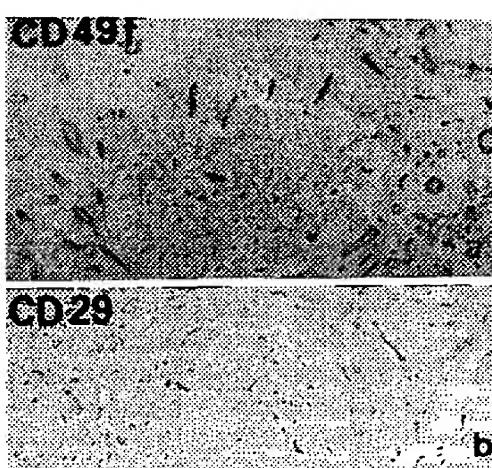


Figure 23 (a) CD49f and (b) CD29 expression in a tonsillar lymphoid follicle (immunoperoxidase method, counterstained with methylgreen).

localized in the LF,⁷⁹⁻⁸² but the follicular localization of other Ca²⁺-binding proteins has yet to be elucidated.

The results of a study on this subject are summarized in Table 8.⁸³ The BLZ, but not the OZ, expressed all the Ca²⁺-binding proteins tested for, the ALZ expressed them all except S-100 protein, whereas the MZ expressed only one, caldesmon, and the reactivity of the DZ was heterogeneous, but S-100 protein was invariably found. These results suggest that the BLZ is rather heavily dependent on Ca²⁺ ions, which may be important for apoptosis during negative cell selection.

Immunoelectron microscopy showed caldesmon expression in a 'belt' along the slender cytoplasmic extensions of FDC (Fig. 24). Both immunohistochemistry and *in situ* hybridization confirmed actin filament proteins and their mRNAs were present on FDC (Fig. 25), implying a close association between caldesmon and actin filaments even in non-muscle cells. Annexin II may be involved in the aggregation of stress fibers,⁸⁴ and some Ca²⁺-binding proteins may be involved in the cytoplasmic extension and FDC adhesion to extracellular fibers.

Cytokine receptors in human tonsillar lymphoid follicles

The exact follicular localization of cytokines is the subject of considerable debate, mainly because of problems with detection techniques.⁸⁵⁻⁸⁸ Therefore, Yamada *et al.* evaluated a cytokine network by detecting their receptors on FDC.⁸⁹

Table 7 Frozen section binding assay of follicular dendritic cells⁷⁷

Pretreatment of antibody	Inhibition (%)
Control	100.0
Anti-CD29 (integrin $\beta 1$)	32.8
Anti-CD49b (VLA- $\alpha 2$)	100.0
Anti-CD49c (VLA- $\alpha 3$)	91.7
Anti-CD49e (VLA- $\alpha 5$)	29.7
Anti-CD49f (VLA- $\alpha 6$)	18.8

VLA, very late activation antigen.

Table 8 Localization of Ca²⁺-binding proteins in secondary lymphoid follicles⁸³

Ca ²⁺ -binding protein	MZ	OZ	DZ	ALZ	BLZ
Calmodulin	-	-	-	++	++
Calbindin-D	-	-	-	++	++
Calcineurin	-	-	\pm	++	++
Caldesmon	+	-	\pm	++	++
Annexin II (Lipocortin II)	-	-	+	++	++
Annexin VI (Lipocortin VI)	-	-	-	++	+
S-100 protein	-	-	++	-	++

MZ, mantle zone; OZ, outer zone; DZ, dark zone; BLZ, basal light zone; ALZ, apical light zone.

-, Negative; \pm , often positive; +, weakly positive; ++, strongly positive.

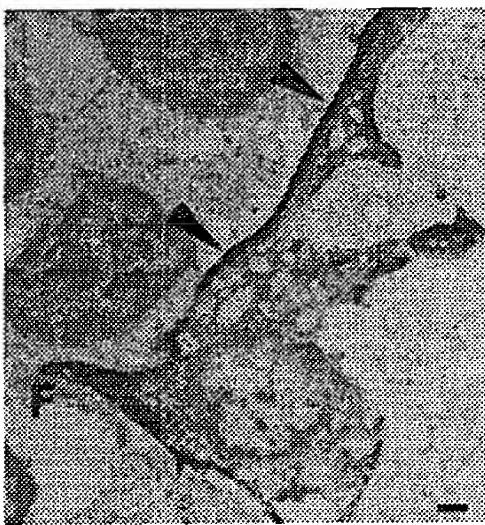


Figure 24 Immunoelectron microscopy features of caldesmon on a FDC. Belt-like expression (arrowheads) is evident at the periphery of the FDC cell body. FDC adhesion to fibers (F) is evident. Bar = 1 μ m.

FDC in the LZ expressed receptors for GM-CSF (α -chain; CDw116), tumor necrosis factor (TNF) (type I; CD120a), interleukin (IL)-1 (type II; CD121b), IL-2 (β -type; CD122), IL-4 (CDw124), IL-6 (CD126), transforming growth factor (TGF)- β (type II) and nerve growth factor (NGF) (Fig. 26, Table 9). FDC in the DZ expressed only IL-2 (α -type; CD25) and NGF receptors, but none of the FDC in the MZ and OZ expressed any cytokine receptors. On the other hand, the only cytokine of those examined in this study expressed by FDC in the LZ was TGF- β . Although a small group of GC cells was labeled with anti-IL-2 and IL-4, very few GC cells expressed cytokine receptors.

These results indicate that FDC themselves do not actively produce and secrete cytokines, but by expressing their receptors, they may present information about cytokines to GC B cells, as is the case with antigen presentation, and may thus regulate migration, selection and differentiation of GC B cells.

Cell cycle analysis of B cells in secondary lymphoid follicles

Immunolabeling studies using antibodies, such as Ki-67, against proliferating cell markers and bromo-deoxyuridine have demonstrated that, in general, the DZ contains dividing centroblasts and the MZ contains resting B cells.^{5,9} Ki-67 is expressed on almost all cells entering the cell cycle. Until now, no studies have been reported in which the cell cycle (phases G0, G1S and G2M) of follicular cells has been analyzed.

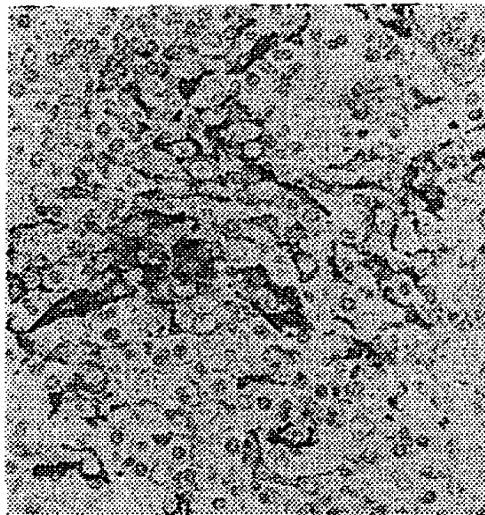


Figure 25 α -Actin expression on FDC in a tonsillar lymphoid follicle (immunoperoxidase method, counterstained with methylgreen).

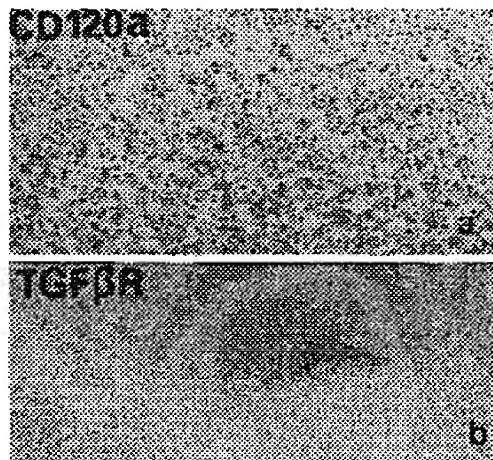


Figure 26 (a) CD120a expression and (b) transforming growth factor- β -receptor expression in the LZ (immunoperoxidase method, counterstained with methylgreen).

Ohrui *et al.* carried out an elaborate analysis of the cell cycle phases of B cells in the five tonsillar follicular zones using immunohistochemistry with antibodies against cyclins and *in situ* hybridization using an S-phase marker, histon H3 (Tables 10–14).⁹¹ The markers for the G2M-phase (cyclin B1 and p34cdc2) and for the S-phase (histon H3 mRNA) were expressed on the majority of DZ cells and some LZ and OZ cells, whereas the markers for pan-cell cycle phases (cyclin E and Ki-67) were detected very frequently on DZ cells and

Table 9 Localization of cytokine receptors in secondary lymphoid follicles⁸⁹

Cytokine receptor	CD code	FDC-expression	MZ	OZ	DZ	BLZ	ALZ
IL-2 receptor α	CD25	-	-	-	+	+	+
GM-CSF receptor	CDw116	+	-	-	-	++	++
TNF receptor I	CD120a	+	-	-	-	++	++
TNF receptor II	CD120b	-	-	-	-	+	+
IL-1 receptor I	CD121a	-	-	-	-	-	-
IL-1 receptor II	CD121b	+	-	-	-	++	++
IL-2 receptor β	CD122	+	-	-	-	++	++
IL-4 receptor	CDw124	+	-	-	-	++	++
IL-6 receptor	CD126	+	-	-	-	++	++
TGF β receptor II		+	-	-	-	\pm	++
NGF receptor		+	-	-	+	+	+

FDC, follicular dendritic cell; MZ, mantle zone; OZ, outer zone; DZ, dark zone; BLZ, basal light zone; ALZ, apical light zone; IL, interleukin; GM-CSF, granulocyte/macrophage-colony stimulating factor; TNF, tumor necrosis factor; TGF, transforming growth factor; NGF, nerve growth factor.

-, Negative; \pm , often positive; +, weakly positive; ++, strongly positive.

Table 10 Immunophenotype of follicular dendritic cells (FDC) in the mantle zone (MZ)

Antigen/Clone	CD code	MZ
FDC-associated antigen		
R4/23		+
Ki-M4		+
Cell cycle marker		
Cyclin E (G1S)		34.2 \pm 7.5
Cyclin B1 (G2M)		3.3 \pm 1.7
Histon H3 (S)		1.8 \pm 1.2
Ca²⁺-binding protein		
Calmodulin		-
Caldesmon		+
Annexin II		-
Annexin VI		-
S-100 protein		-
Intermediate filament		
α -actin		-
β -actin (mRNA)		-
Vimentin		+
Desmin		-
Complements		
CR & FcR		-
CR2	CD21	+
Fc ϵ RIII	CD23	+
CR1	CD35	+
Adhesion molecule		
s-Le ^a	CD15s	+
MAB89	CD40	+
VLA- α 3	CD49c	-
VLA- α 5	CD49e	-
VLA- α 6	CD49f	-
ICAM-3	CD50	-
ICAM-1	CD54	-
VCAM-1	CD106	+
Cytokine receptors		
GM-CSFR	CDw116	-
TNFRI	CD120a	-
IL-1RII	CD121b	-
IL-2R β	CD122	-
IL-4R	CDw124	-
IL-6R	CD126	-
TGF- β RII		-

-, Negative; +, weakly positive.

Table 11 Immunophenotype of follicular dendritic cells (FDC) in the outer zone (OZ)

Antigen/Clone	CD code	OZ
FDC-associated antigen		
R4/23		+
Li-M4		+
Cell cycle marker		
Cyclin E (G1S)		52.7 \pm 8.2
Cyclin B1 (G2M)		22.5 \pm 9.7
Histon H3 (S)		14.9 \pm 3.8
Ca²⁺-binding protein		
Calmodulin		-
Caldesmon		-
Annexin II		-
Annexin VI		-
S-100 protein		-
Intermediate filament		
α -actin		-
β -actin (mRNA)		-
Vimentin		+
Desmin		+
Complements		
CR & FcR		-
CR2	CD21	+
Fc ϵ RII	CD23	-
CR1	CD35	+
Adhesion molecule		
s-Le ^a	CD15s	+
MAB89	CD40	+
VLA- α 3	CD49c	-
VLA- α 5	CD49e	-
VLA- α 6	CD49f	-
ICAM-3	CD50	-
ICAM-1	CD54	-
VCAM-1	CD106	+
Cytokine receptors		
GM-CSFR	CDw116	-
TNFRI	CD120a	-
IL-1RII	CD121b	-
IL-2R β	CD122	-
IL-4R	CDw124	-
IL-6R	CD126	-
TGF- β RII		-

-, Negative; +, weakly positive.

Table 12 Immunophenotype of follicular dendritic cells (FDC) in the dark zone (DZ)

Antigen/Clone	CD code	DZ
FDC-associated antigen		
R4/23		+
Ki-M4		+
Cell cycle marker		
Cyclin E (G1S)		54.5 ± 6.6
Cyclin B1 (G2M)		36.7 ± 14.5
Histon H3 (S)		21.2 ± 6.6
Ca²⁺-binding protein		
Calmodulin		-
Caldesmon		+
Annexin II		+
Annexin VI		-
S-100 protein		++
Intermediate filament		
α-actin		-
β-actin (mRNA)		-
Vimentin		+
Desmin		-
Complements		
CR & FcR		
CR2	CD21	+
FcεRII	CD23	-
CR1	CD35	-
Adhesion molecule		
s-Le ^x	CD15s	+
MAB89	CD40	+
VLA-α3	CD49c	-
VLA-α5	CD49e	-
VLA-α6	CD49f	-
ICAM-3	CD50	+
ICAM-1	CD54	±
VCAM-1	CD106	+
Cytokine receptors		
GM-CSFR	CDw116	-
TNFRI	CD120a	-
IL-1RII	CD121b	-
IL-2Rβ	CD122	-
IL-4R	CDw124	-
IL-6R	CD126	-
TGF-βRII		-

-, Negative; ±, often positive; +, weakly positive; ++, strongly positive.

quite often on cells in the other four zones. Furthermore, FDC-lymphocyte clusters composed of one or more FDC and 10–30 lymphocytes were isolated from secondary LF of paraformaldehyde-fixed tonsillar tissues. The clusters contained numerous Ki-67⁺ lymphocytes, possibly in the G1S phase, because the clusters contained very few G2M-phase lymphocytes. Contrary to expectations, G2M-phase cells were not rarely found in the OZ, suggesting that this zone may not only be a cellular pathway in the LF but also a cell proliferation site. FDC-lymphocyte clusters contained less lymphocytes in the G2M phase than in other phases, implying that the clusters may not provide a site for GC cell division, but rather one for cell selection and differentiation.

Table 13 Immunophenotype of follicular dendritic cells (FDC) in the basal light zone (BLZ)

Antigen/Clone	CD code	BLZ
FDC-associated antigen		
R4/23		++
Ki-M4		++
Cell cycle marker		
Cyclin E (G1S)		52.5 ± 6.8
Cyclin B1 (G2M)		19.8 ± 6.6
Histon H3 (S)		13.5 ± 2.5
Ca²⁺-binding protein		
Calmodulin		++
Caldesmon		++
Annexin II		++
Annexin VI		++
S-100 protein		++
Intermediate filament		
α-actin		±
β-actin (mRNA)		++
Vimentin		++
Desmin		-
Complements		
CR & FcR		
CR2	CD21	++
FcεRII	CD23	±
CR1	CD35	++
Adhesion molecule		
s-Le ^x	CD15s	+
MAB89	CD40	++
VLA-α3	CD49c	+
VLA-α5	CD49e	+
VLA-α6	CD49f	+
ICAM-3	CD50	++
ICAM-1	CD54	++
VCAM-1	CD106	++
Cytokine receptors		
GM-CSFR	CDw116	++
TNFRI	CD120a	++
IL-1RII	CD121b	+
IL-2Rβ	CD122	+
IL-4R	CDw124	+
IL-6R	CD126	+
TGF-βRII		+

-, Negative; ±, often positive; +, weakly positive; ++, strongly positive.

In vitro characteristics of isolated FDC-lymphocyte clusters

Some methods of isolating FDC and FDC-lymphocyte clusters have been reported^{28,92} and they are useful for analyzing the characteristics of FDC and the cellular interactions between FDC and lymphocytes in the FDC-lymphocyte clusters.⁹³

Similar to the findings *in vivo*, FDC in clusters cultured for 1 week have lots of cytoplasmic extensions.^{94,95} Further culture resulted in the apoptotic death of lymphocytes and at the end of 2 weeks only FDC survived and they had an umbrella-like appearance (Fig. 27).⁹⁴ These FDC survived for

Table 14 Immunophenotype of follicular dendritic cells (FDC) in the apical light zone (ALZ)

Antigen/Clone	CD code	ALZ
FDC-associated antigen		
R4/23		++
Ki-M4		++
Cell cycle marker		
Cyclin E (G1S)		54.3 ± 7.2
Cyclin B1 (G2M)		19.0 ± 7.8
Histon H3 (S)		6.6 ± 1.5
Ca²⁺-binding protein		
Calmodulin		++
Caldesmon		++
Annexin II		++
Annexin VI		++
S-100 protein		-
Intermediate filament		
α-actin		±
β-actin (mRNA)		++
Vimentin		++
Desmin		-
Complements		
CR & FcR		++
CR2	CD21	++
FcεRII	CD23	++
CR1	CD35	++
Adhesion molecule		
s-Le ^x	CD15s	++
MAB89	CD40	++
VLA-α3	CD49c	+
VLA-α5	CD49e	+
VLA-α6	CD49f	+
ICAM-3	CD50	++
ICAM-1	CD54	++
VCAM-1	CD106	++
Cytokine receptors		
GM-CSFR	CDw116	++
TNFRI	CD120a	++
IL-1RII	CD121b	+
IL-2Rβ	CD122	+
IL-4R	CDw124	+
IL-6R	CD126	+
TGF-βRII		++

-, Negative; ±, often positive; +, weakly positive; ++, strongly positive.

more than 150 days in the absence of added cytokines and from the early phase of cluster culture FDC began to dedifferentiate and gradually lost their relatively specific characteristics, including their immunohistochemical and cytochemical markers. One of the most striking FDC functions *in vitro* was emperipoleisis: lymphocytes added to FDC in culture were enveloped by long cytoplasmic FDC extensions that expressed α-smooth muscle actin.⁸⁵

In summary, FDC isolated from tonsils can survive in culture for a long time without any additional cytokines, but under these conditions, they dedifferentiate rapidly. *In vitro* FDC envelop lymphocytes with their cytoplasmic extensions and inhibit the apoptotic death of lymphocytes.

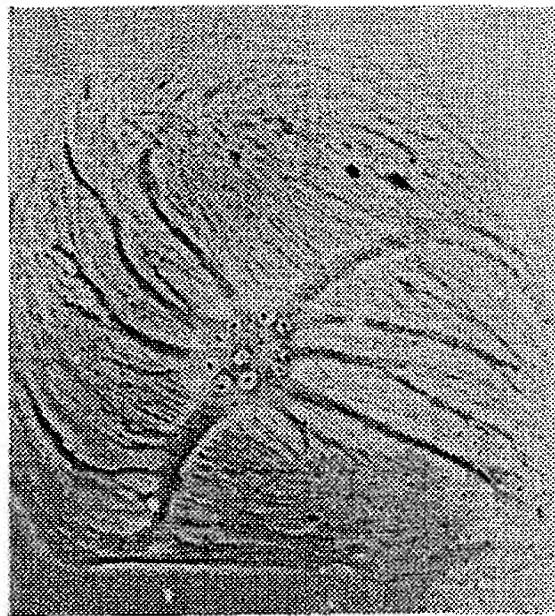


Figure 27 Umbrella-like features of a cultured FDC. Note the multinucleation.

SUMMARY OF FDC MORPHOLOGY AND FUNCTION IN REACTIVE LYMPHOID HYPERPLASIA

The characteristics of FDC in the five tonsillar follicular zones are summarized (Fig. 28).

B cells expressing CD62L (L-selectin) actively pass through postcapillary venules and migrate into T-cell-dependent areas out of the LF. Some of these cells migrate to the crescent-shaped MZ, which is composed of FDC extending their slender cytoplasmic processes, and mIgM⁺ and mIgD⁺ recirculating, small resting B cells.⁵ When these small B cells reach the top of the MZ (in the central portion of the crescent-shaped MZ), they are entrapped by FDC to form FDC-lymphocyte clusters (Table 10). IC trapping occurs in this zone and it seems likely that antigenic stimuli cause lymphocytes in the clusters to secede and migrate through the OZ to the DZ.

The OZ contains a variety of cell types, including CD4⁺ T cells, which may be associated with B cell stimulation and migration, and the zone plays a role as a cellular pathway.⁴¹⁻⁴³ Cell cycle data from Ohri *et al.*⁹¹ indicated that this zone, as well as the DZ, contains relatively numerous B cells in the G2M phase (Table 11).

The DZ contains FDC with less well-developed labyrinth-like structures than these in the LZ, centroblasts and occasional TBM (Table 12). FDC in this zone wrap centroblasts loosely with their cytoplasmic extensions, but do not form

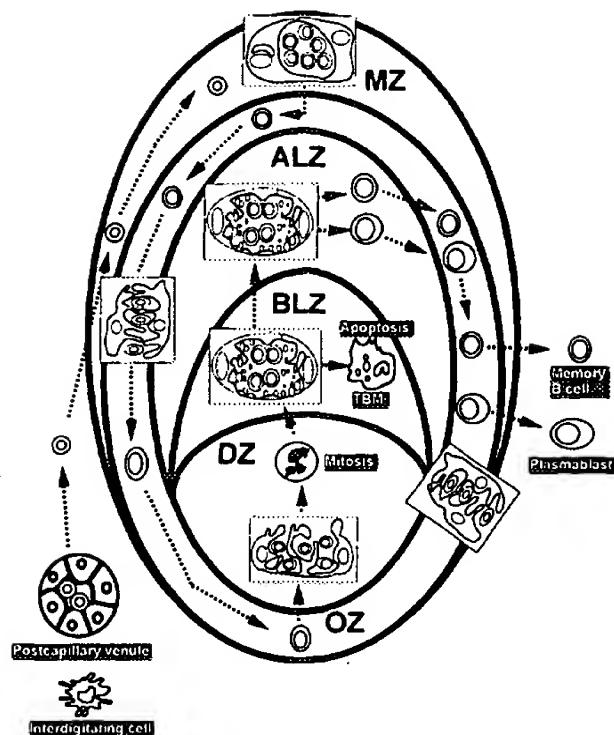


Figure 28 Functions of tonsillar secondary lymphoid follicles. MZ, mantle zone; ALZ, apical light zone, BLZ, basal light zone; DZ, dark zone; OZ, outer zone; TBM, tingible body macrophage.

clusters. Centroblasts divide extensively with clonal expansion and somatic mutation in the variable portion of Ig-region gene.⁹⁸⁻¹⁰² When centroblasts in the DZ migrate to the BLZ, they become large non-dividing centrocytes, which express mlg and leukocyte-function-associated antigen-1.⁵

The BLZ contains numerous FDC with well-developed labyrinth-like structures and villous cytoplasmic extensions (Table 13). Entrapped antigens settle on the FDC surfaces as IC, and free antigens lead to complement activation. Free antigens are essential for positive and negative selection of centrocytes,⁵³ and it is likely that cell selection may be carried out effectively in the FDC-lymphocyte clusters, although apoptotic centrocytes are rarely found in them. If antigen receptors on centrocytes have low affinities for free antigens, these centrocytes are released immediately from the cluster to die by apoptosis and are finally phagocytosed by TBM. Centrocytes expressing membrane Ig with high affinities for free antigens are positively selected, survive and migrate to the ALZ to differentiate into memory B cells and plasmablasts under regulation of some cytokines secreted mainly by GC T cells.

The ALZ contains numerous FDC that produce and secrete soluble CD23, which is essential for B cell differentiation

(Table 14). As outlined before, Yamada *et al.* have demonstrated that FDC express some cytokine receptors and FDC may simultaneously transfer information about cytokines to B cells simultaneously when the latter present antigens.⁶⁹

FDC form not only FDC-lymphocyte clusters, but also the three-dimensional structure of the secondary LF. This structure is maintained by additional important factors such as the desmosome-like structures between neighboring FDC, expression of laminin and fibronectin receptors on FDC in order to bind to extracellular matrices, expression of actin filaments in FDC and expression of caldesmon in FDC to bind to or be interlaced with extracellular fibers.

After differentiation in the ALZ, memory B cells and plasmablasts again pass through the OZ, leave the LF, migrate via the efferent lymphatics into the blood stream and, finally, take up residence in the bone marrow, the lymphatic tissues or inflammatory sites.

FDC in each of these five zones play important roles in the series of immunological events involving B cells, including cell migration, proliferation, selection and differentiation.

FOLLICULAR DENDRITIC CELLS IN DISEASE CONDITIONS

Autoimmune diseases

As described earlier, the LZ is the site governing IC trapping and retention processes, which are mediated by complement activation. Hashimoto's thyroiditis and rheumatoid arthritis are representative autoimmune diseases and the affected tissues of patients with these conditions invariably show dense lymphocytic infiltration, often with secondary LF and T-cell-dependent areas including interdigitating cells.^{103,104} Draining lymph nodes, especially in patients with rheumatoid arthritis, are sometimes swollen and the normal tissue is replaced largely by reactive follicular hyperplasia containing secondary LF (Fig. 29) and the LZ in such hyperplastic secondary LF contain thyroglobulin or rheumatoid factor proteins well known to be autoantigens (Table 15).¹⁰⁵⁻¹⁰⁷ It seems likely that the thyroglobulin comes from colloid follicles destroyed by chronic inflammation, binds to anti-thyroglobulin autoantibodies, and then is trapped in the GC. Immunoelectron microscopy has revealed thyroglobulin and rheumatoid factor on the surface of the labyrinth-like structures of FDC.¹⁰⁸

The LZ contains immunoglobulins, which possibly are autoantibodies, as well as complement components and thyroglobulin or rheumatoid factor autoantigens, revealing that autoantigens are trapped and retained like IC or if they are free lead to complement activation. Because the IC in the LZ plays an essential role in the production of memory B

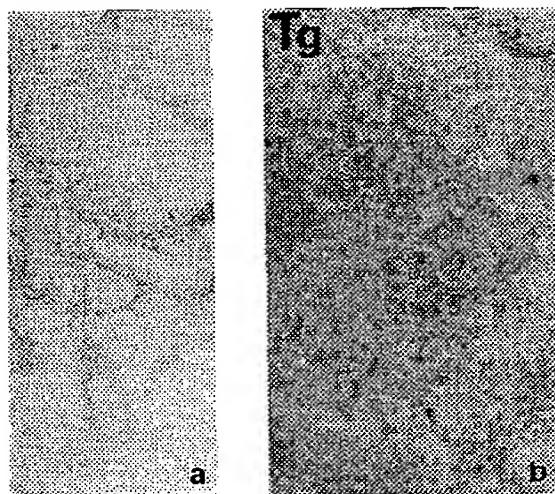


Figure 29 (a) HE staining and (b) thyroglobulin (Tg) localization in the follicular LZ of a patient with Hashimoto's thyroiditis (immuno-peroxidase method, counterstained with methylgreen).

cells and plasmablasts that become autoantibody-forming plasma cells, it seems reasonable to conclude that the IC may be one of the factors controlling the establishment and prolongation of autoimmune diseases and in patients with these disease conditions, the FDC may be in a hyperfunctional state.

Disappearance of secondary lymphoid follicles in Kimura's disease and acquired immunodeficiency syndrome

The LF undergoing destruction show a variety of histological features, including regressive and progressive transformation, dissolution and necrosis of GC, and follicle lysis.¹⁰⁸ The mechanisms responsible for LF loss and follicle lysis in Kimura's disease and AIDS have been compared histologically and immunohistochemically.

It is well known that the combination of florid GC hyperplasia and increased numbers of postcapillary venules, eosinophilic infiltration and sclerosis in the lymph node paracortices is characteristic of Kimura's disease.^{108,109} These patients demonstrate peripheral eosinophilia and elevated serum IgE levels,¹¹⁰ and loss of secondary LF in primary lesions and the draining lymph nodes are not infrequent findings. Secondary LF in both primary and secondary lesions are often eroded and destroyed gradually by the mass of extrafollicular lymphocytic infiltrate with or without eosinophils. FDC immunostaining reveals disintegrated LF with a variety of appearances from moth-eaten to marked atrophy with only laminated FDC remaining (Fig. 30).

Table 15 Localization of immune complexes in the germinal centers in autoimmune diseases

Antigen	Hashimoto's disease	Rheumatoid arthritis
Immunoglobulin		
IgG	+	+
IgM	++	++
IgA	+	+
IgE	-	-
Complement		
C1q	++	++
C3d	++	++
C9	+	+
Properdin	+	+
CD21	++	++
CD35	++	++
Specific antigen		
Thyroglobulin	+	-
Rheumatoid factor	-	+

-, Negative; +, weakly positive; ++, strongly positive.

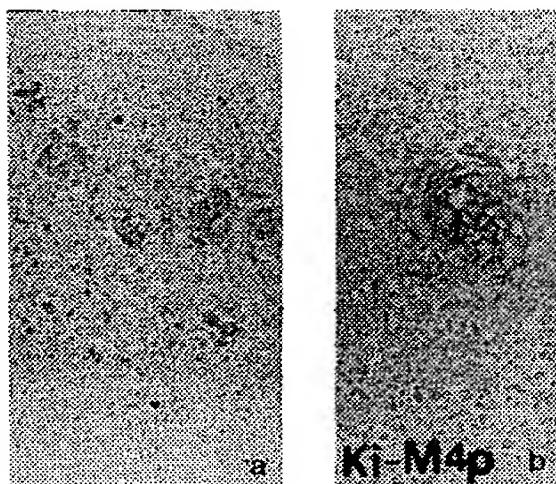


Figure 30 Immunostaining with an anti-FDC antibody (Ki-FDC-M4p) of a LF from a patient with Kimura's disease. Note the (a) moth-eaten and (b) atrophic appearance (immunoperoxidase method, counterstained with hematoxylin).

In the clinical stage of persistent generalized lymphadenopathy in AIDS, lymphoid hyperplasia with secondary LF is prominent.¹¹¹ As in Kimura's disease, in the later stages of AIDS hyperplastic LF are eroded by a mass of MZ lymphocytes, revealing so-called 'follicle lysis', which is one of the characteristic histological features of AIDS.¹¹² Human immunodeficiency virus-related lymphadenopathy is sometimes accompanied by loss of the MZ, paracortical hyperplasia and regressive transformation of GC.¹⁰⁸ Immunostaining of FDC has also revealed distinct follicle lysis in a moth-eaten

pattern¹¹³ and human immunodeficiency virus core protein p24 was detected immunohistochemically on FDC in LF undergoing destruction.¹¹⁴

Although follicle destruction is a characteristic histological feature of both Kimura's disease and AIDS, we propose the possibility that there are two different destruction patterns. Type I (follicle loss or necrosis showing atrophic LF) and type II (follicle lysis showing a moth-eaten pattern), are found in the former, whereas type II follicle lysis predominates in AIDS. It has been speculated that type I (atrophic) follicle loss may also be observed in LF of patients affected by infectious mononucleosis, overwhelming childhood infections,¹⁰⁸ anti-cancer agents and steroids, in irradiated LF and in LF in cases of starvation and aging. Type II follicle lysis showing a moth-eaten pattern may be observed in LF of patients with infectious mononucleosis, atypical lymphoproliferative disorders, Hodgkin's disease and non-Hodgkin's malignant lymphomas, as well as in AIDS.

Follicular lymphoma

It is now well known that not only follicular, but also diffuse lymphomas have a variety of FDC meshworks from well-preserved to fairly destructive patterns and in some lymphomas there is still a functional relationship between FDC and neoplastic lymphoma cells similar to that observed in non-neoplastic LF.^{115,116} FDC in lymphomas express CR3 (CD11b), CD49e, CD49f and CD54, whereas centroblastic–centrocytic lymphoma cells express CD11a/CD18 and CD54.^{117–119} In the leukemic stage all the adhesion molecules on lymphoma cells disappear.

Maeda *et al.* have evaluated the immunophenotype of FDC and the capacity of LF in follicular lymphomas to trap IC *in vitro*.¹²⁰ Electron microscopy revealed varying numbers of

FDC in neoplastic follicles and like those in non-neoplastic LF, the FDC were positive for some FDC associated markers, including R4/23, Ki-M4, DF-DRC-1, CD35 and CD21 (Table 16). Binding of IC (rabbit peroxidase-anti-peroxidase complex) to fresh tissue sections was found to be limited to neoplastic follicles and to reactive LF in the positive controls (tonsillar tissues from patients with chronic tonsillitis) (Fig. 31). These results indicate that, like those in reactive LF, FDC in the neoplastic follicles of follicular lymphomas express CD21 and CD35, which are essential for IC trapping, *in vitro*.

Follicular dendritic cell tumors

Up to now, only eight cases of FDC tumors have been reported in the English-speaking literature.^{121–123} This tumor

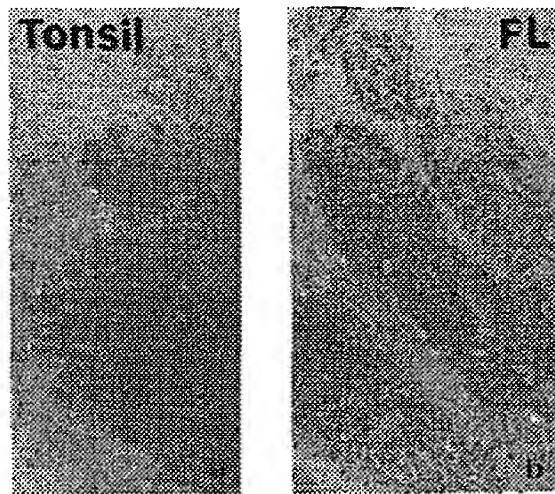


Figure 31 Follicular binding *in vitro* of immune complex in frozen tissue sections of (a) tonsil (control) and (b) follicular lymphoma (counterstained with methylgreen).

Table 16 Heterogeneity of follicular dendritic cells in follicular lymphomas (FL)

Case	Control (Chronic tonsillitis)	FL, mixed (cb/cc)	FL, small (cb/cc)	FL, mixed (cb/cc)	FL, mixed (cb/cc)	FL, small (cb/cc)
R4/23	++	++	++	+	++	+
Ki-M4	++	++	++	++	+	+
Bu10	++	+	++	+	±	±
DF-DRC1	++	++	+	+	–	–
X11	++	+	+	+	±	–
CD21	++	++	+	ND	+	+
CD23	++	+	ND	ND	+	Tumor cells
CD35	++	++	++	++	++	
CD55	++	+	++	+	–	
NGFR	++	++	++	++	±	
<i>In vitro</i> IC trapping	++	++	++	+	+	

IC, immune complex; ND, not done; NGFR, nerve growth factor receptor.

–, Negative; ±, often positive; +, weakly positive; ++, strongly positive.

is very rare, like the malignant neoplasms of interdigitating cells in T-cell dependent areas,¹²⁴⁻¹²⁶ and the majority of the cases were clinically and histologically malignant, although some were of a benign nature. No cases in Japan have ever been reported, but we have had the chance to observe tissues from three of the reported cases.¹¹²⁻¹¹⁴ These tumors had a microscopic appearance grossly similar to that of fibrohistiocytic tumors, demonstrating storiform or interlacing

bundle-like patterns (Fig. 32). The tumor cells had one or two elliptical, spindle-shaped or irregular nuclei with occasional prominent nucleoli. Electron microscopy showed that these cells were similar to non-neoplastic FDC, having slender cytoplasmic extensions, varying numbers of clearly recognizable labyrinth-like structures and numerous desmosome-like junctions (Fig. 33). The tumors frequently contained non-neoplastic intermingled lymphocytes. Examination of paraffin

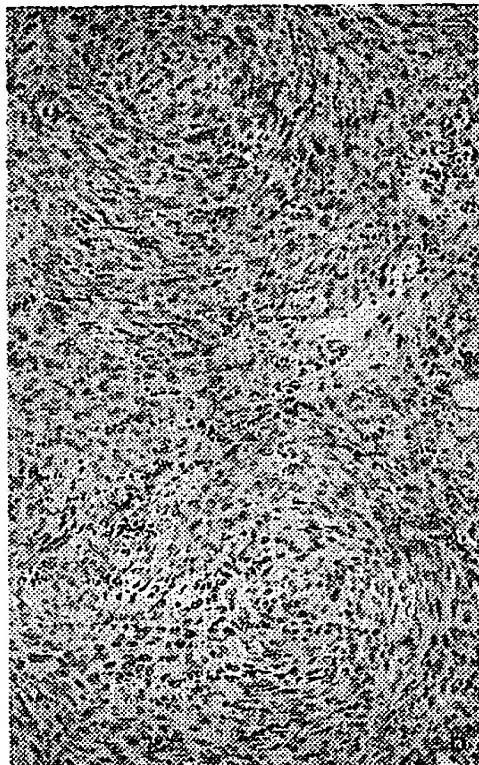
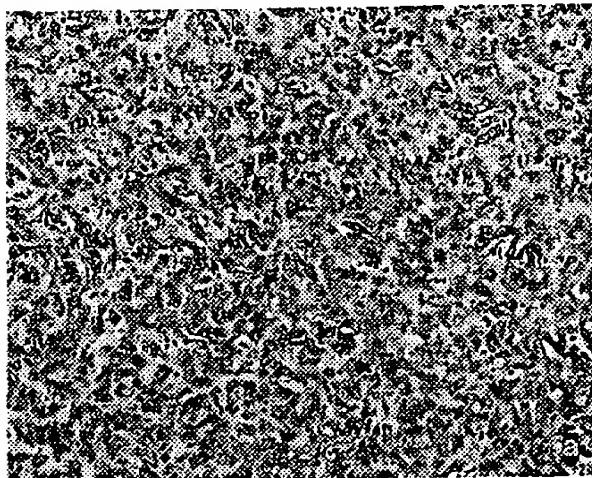


Figure 32 Light microscopic features of a FDC tumor. (a) Note the fibrohistiocytic tumor-like appearance¹¹⁴ and (b) follicle-like appearance¹¹³ (HE).

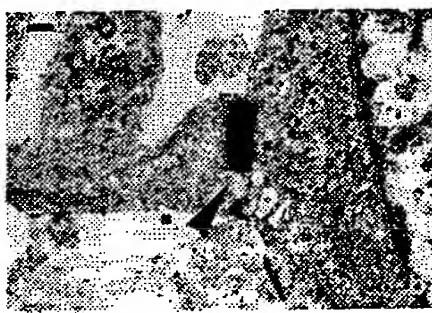
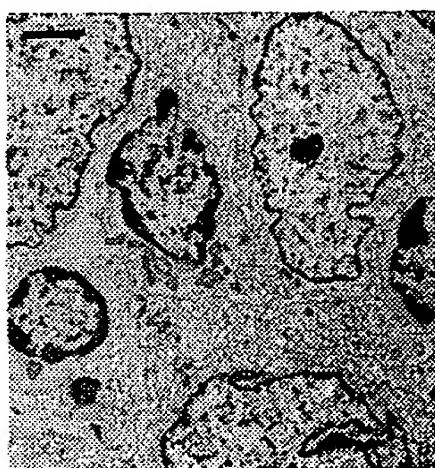


Figure 33 Transmission electron microscopic features of a FDC tumor.¹¹² (a) Note the close resemblance of the neoplastic cells to non-neoplastic FDC with abundant labyrinth-like structures. Asterisks indicate intermingled lymphocytes. Bar = 1 μ m. (b) Note the distinct desmosome-like structure (arrowhead). Bar = 10 μ m.

sections revealed that neoplastic FDC shared some markers in common with non-neoplastic FDC, including Ki-M4p (Fig. 34), CD 21 and CD23.

These results indicate that useful and significant histological findings for diagnosing FDC tumors include a light microscopic appearance like fibrohistiocytic tumors, labyrinth-like structures desmosome-like junctions on electron microscopy, and a positive reaction with anti-FDC antibodies.

CELLULAR ORIGIN OF FOLLICULAR DENDRITIC CELLS

Until now, a great deal of data about the origin of FDC has been obtained using a variety of research approaches,

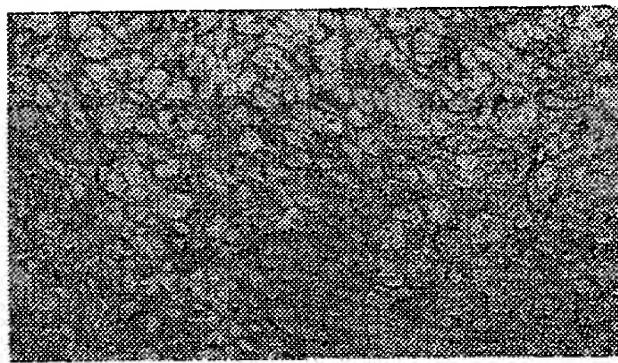


Figure 34 FDC-M4p immunostaining of a FDC tumor¹²¹ (immuno-peroxidase method, counterstained with methylgreen).

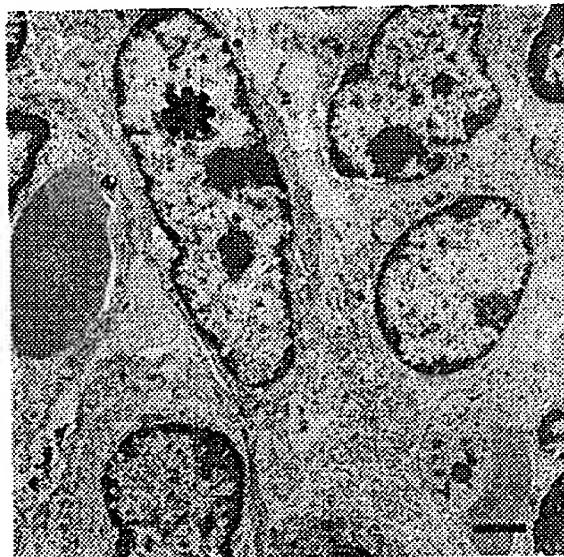


Figure 35 Transmission electron photomicrograph showing fibrohistiocytoid cell (*) in reactive lymphadenitis. Bar = 1 μ m.

and studies identifying some characteristics of FDC, including their morphological, immunophenotypic, ontogenetic and genetic aspects, have been published.^{34,35} However, no consensus has been reached and there are two major hypotheses: (i) the bone marrow-derived and (ii) stromal cell-derived (FRC in lymphatic tissues) theories.

Next we present recent data based on bone marrow chimerae in severe combined immunodeficiency disease (SCID) mice and new ultrastructural findings.

Bone marrow chimerae in SCID mice

Transplantation of bone marrow cells from C3H mice and Wistar rats into SCID mice has revealed that the FDC were not derived from bone marrow cells.²⁵ In that study FDC in secondary LF in SCID mice reconstructed with C3H mouse cells were immunohistochemically positive for H-2^a antigen, a marker of SCID mice cells, but not for H-2^k antigen, a marker of C3H mice cells. However, H-2^k antigen was expressed on neighboring lymphocytes in secondary LF, and FDC in SCID mice LF reconstructed with rat bone marrow cells were also positive for FDC-M1, a marker of murine FDC. These data indicate that the reconstructed LF in SCID mice were composed of FDC derived from SCID mice and donor lymphocytes.

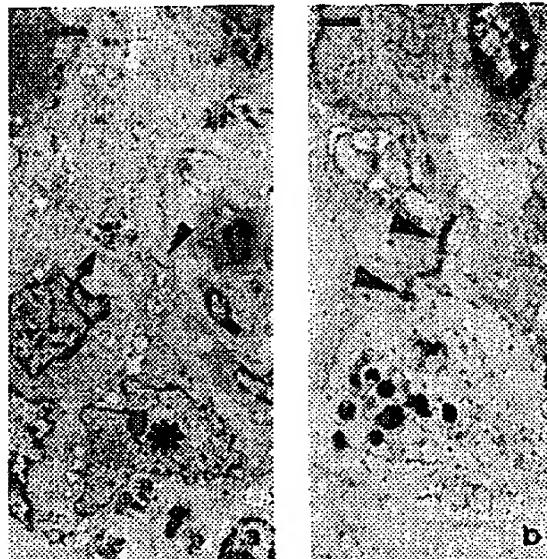


Figure 36 Electron photomicrograph showing cells (*) intermediate between FDC and fibrohistiocytoid cells with dense bodies (arrow) and desmosome-like junctions (arrowheads). (a) Bar = 1 μ m. (b) High-power view of (a); bar = 4 μ m.

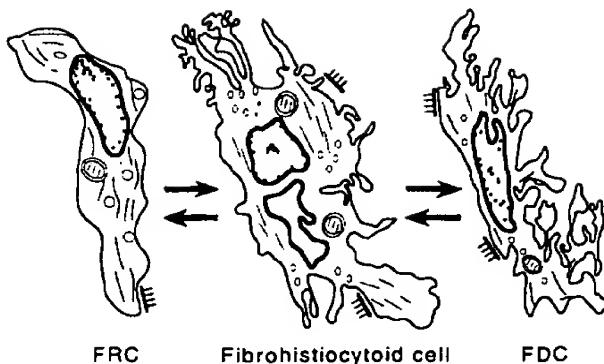


Figure 37 Hypothesis of the origin of FDC. FRC, fibroblastic reticulum cell; FDC, follicular dendritic cell.

Similarities of follicular dendritic and fibrohistiocytoid cells

It has been reported that fibrohistiocytoid (FH) cells in granulation tissues in various sites are metamorphosed fibroblasts that assume a histiocytic nature to some extent¹²⁷ and it has been demonstrated that malignant fibrous histiocytomas may be derived from this type of cell. The ultrastructural characteristics of FH cells include an elliptical, often irregular nucleus, fragmented rough endoplasmic reticulum and often dense bodies. Briefly, the light microscopy and immunohistochemical natures of this cell type are intermediate between those of fibroblasts and histiocytes. It was also confirmed that FH cell formation from dermal fibroblasts can be induced *in vitro*.

Our present ultrastructural study (unpubl. data) demonstrated that certain cell types in reactive LF closely resemble FH cells (Fig. 35), which have slender cytoplasmic extensions and distinctive desmosome-like junctions. Communication between typical FH cells via these junctions was occasionally found. With further elaborate observation we could demonstrate FDC with typical labyrinth-like structures and dense bodies closely resembling those in FH cells (Fig. 36), revealing morphological similarities between FDC and FH cells in LF.

These studies indicate that FDC may be derived from FRC, not bone marrow cells, and FH cells may be an intermediate cell type between FDC and FRC in lymphatic tissues (Fig. 37).

SUMMARY

The summary of the present review is as follows.

(1) In reactive conditions, FDC in each of the five follicular zones have distinct ultrastructural features, reflecting the

different three-dimensional structures and functions of these zones.

(2) The FDC framework may be supported by some characteristic factors, including desmosome-like junctions between FDC and the expression of fibronectin and laminin receptors and caldesmon on FDC.

(3) The BLZ may be rather heavily dependent on Ca^{2+} ions, which may be important for negative GC cell selection.

(4) FDC, especially in the LZ, express various cytokine receptors, but produce only one cytokine, TGF- β . FDC themselves do not appear to produce and secrete cytokines actively, but they may present information about them to GC B cells via their cytokine receptors.

(5) The OZ may not only be a cellular pathway in the LF, but may also provide a site for GC B cell proliferation. The FDC-lymphocyte cluster is not the site of GC B cell division.

(6) In patients with autoimmune diseases, such as Hashimoto's thyroiditis and rheumatoid arthritis, FDC may be in a hyperfunctional state, whereas those in patients with immunosuppressive disorders, such as Kimura's disease and AIDS, may be in a dysfunctional state.

(7) Some findings, including a fibrohistiocytic tumor-like appearance, labyrinth-like structures, desmosome-like junctions and immunostaining with anti-FDC antibodies are useful for diagnosing FDC tumors.

(8) FDC may be derived from FRC in lymphatic tissues rather than bone marrow cells.

Although the secondary LF in tonsillar tissues of patients with chronic tonsillitis are maximally enlarged, their greatest diameter is less than 5 mm, but they engage in fairly advanced functions. Each of the five tonsillar follicular zones builds up a distinct three dimensional structure and plays its role in sequence. It is no exaggeration to say that the LF is a finely tuned and unified structure that performs the sequential events of B cell proliferation, cellular selection and differentiation and the FDC in secondary LF play a central role in controlling these immunological events.

Further functions and processes in the secondary LF remain to be elucidated. It is necessary to do so to understand the role of LF in chronic inflammation and the causative mechanisms involved in autoimmune diseases, AIDS and anti-cancer immunity. We here emphasize that the FDC is a 'school' and the reticular meshwork of the FDC is a cradle for follicular B cells.

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